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For: **METHOD FOR THE IDENTIFICATION OF ANTIGENIC PEPTIDES**

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Europe	02022223.8	October 2, 2002

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Method for the identification of antigenic peptides associated to diseases

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Method for the identification of antigenic peptides associated to diseases

The present invention relates to methods useful for isolating antigenic peptides from a limited quantity of cells or bodily fluid from a mammalian organism in an amount

5   sufficient to determine their sequence and identity. Therefore this invention relates to methods for identifying novel disease-associated antigens, e.g. tumor antigens and antigens involved in autoimmune diseases, to be utilized for diagnostic or therapeutic purposes. The methods of the present invention can also be utilized for controlling the quality of vaccines. More specifically, the methods of the invention can be used for

10 determining the sequence of antigenic peptides presented via peptide receptors of dendritic cells which are the most important antigen presenting cells of the body and valuable tools for vaccination.

15 Pathological conditions, such as infectious diseases, autoimmune disorders or cancer, can be distinguished from healthy conditions by the expression of disease-specific molecules. In particular, proteins which are newly expressed, mutated or aberrantly expressed, can be utilized as markers for the respective malignancy.

A potent class of markers serving as both diagnostic and therapeutic tools, are

20 protein fragments or peptides bound to molecules of the major histocompatibility complex (MHC). In humans, MHC molecules are termed human leukocyte antigens (HLA). HLA-associated peptides are short, encompassing 9-25 amino acids (Kropshofer, H. & Vogt, A.B., Immunol Today 18 (1997) 77-82). On the one hand, these peptides are

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derived from self-proteins in order to establish self-tolerance. On the other hand, HLA-associated peptides are derived from foreign proteins of viral, fungal or bacterial origin in order to fight foreign invaders. Through activation of specialized immune cells, named T lymphocytes (short: T cells), HLA-peptide complexes are indispensable for mounting a

5 cellular or humoral immune response. Particular self-peptides, denoted autoantigenic peptides, are erroneously recognized by autoaggressive T cells giving rise to autoimmune diseases. Conversely, the lack of T cell recognition of self-peptides derived from tumor-specific antigens, contributes to immune evasion and progressive growth of tumors (Boon, T. et al., Ann Rev Immunol. 12 (1994) 337-265). Hence, increasing our knowledge about

10 disease-associated marker peptides would be of considerable importance in tumor immunology and autoimmunity.

With regard to their function, two classes of MHC-peptide complexes can be distinguished (Germain, R., Cell 76 (1994) 287-299): (i) MHC class I-peptide complexes can be expressed by almost all nucleated cells in order to attract CD8+ cytotoxic T cells

15 which lyse infected cells or tumor cells, (ii) MHC class II-peptide complexes are constitutively expressed only on so-called antigen presenting cells (APCs), such as B lymphocytes, macrophages or dendritic cells (DCs). In particular, DCs have the capacity to prime CD4+ T helper cells (Banchereau, J. & Steinman, R.M., Nature 392 (1998) 245-254). Moreover, DCs can be licensed to optimally activate cytotoxic CD8+ T cells: this is

20 accomplished through prior interaction of their MHC class II-peptide complexes with CD4+ T helper cells (Ridge, T. et al., Nature 393 (1998) 474-478). Thus, peptides presented by MHC class II molecules on DCs play a superior role in the pathogenesis of diseases involving T cell-driven immune responses.

The apparent role of DCs in initiating immune responses has stimulated attempts to

25 exploit DCs as vaccines, in particular against cancer (Dallal, R.M. & Lotze, M.T., Curr Opinion Immunol 12 (2000) 583-588). A key advance was the invention of techniques for differentiation of DCs in vitro from different sources including peripheral blood, e.g. adherent monocytes, or bone marrow-derived CD34+ stem-cell precursors. DCs differentiated and activated in vitro can be used for vaccination of cancer patients after co-

30 culture with tumor cell-derived antigens or by employing analogous techniques. Pilot dendritic cell vaccination studies have successfully induced specific anticancer responses including clinical responses (Timmermann, J.M. & Levy, R., Ann Rev Medicine 50 (1999) 507-529; Nestle, F.O., et al., Nature Medicine 7 (2001) 761-765).

DC-based cancer cell vaccines comprise DCs pulsed with normal or gene-modified

35 cancer cells, cancer cell lysates, cancer cells fused to DCs or cancer cell-derived heat shock protein- (Hsp-) peptide complexes. The rationale behind the latter technique is that Hsps derived from tumor cells carry tumor-specific peptides which are efficiently transferred

onto MHC molecules of DCs. These DCs finally prime cytotoxic T cells with anti-tumor reactivity which leads to the eradication of tumors in mice (Srivastava, P.K., et al., PNAS 83 (1986) 3407-3411; Suto, R., et al., Science 269 (1995) 1585-1588; Binder, R.J. et al, Nature Immunol. 1 (2000) 151-162).

5   The advantage of all these approaches is that no knowledge about the identity of tumor antigens is necessary. The disadvantage is that the identity of tumor markers remains unknown and the copy number of individual HLA-bound tumor peptides is often too low to induce long-lasting anti-tumor T cell responses.

Vaccines based on the identification of cancer antigens include DCs primed with
10   naked DNA, recombinant adeno- or vaccinia viruses, natural or recombinant proteins purified from the respective tumor cells or synthetic analogs of tumor peptides. The advantage of pulsing DCs with antigenic tumor peptides rather than with genetic or protein precursors is that peptides can be loaded directly onto MHC molecules of DCs without further processing.

15   During the past decade, numerous peptides derived from tumor marker proteins and restricted by MHC class I molecules have been identified. They are grouped into four categories: cancer-testes antigens, melanoma-melanocyte differentiation antigens, mutated antigens and non-mutated shared antigens over-expressed on tumors. In several clinical pilot vaccination studies, DCs from melanoma patients were pulsed with cocktails of
20   melanoma peptides which, as yet, were exclusively HLA class I-restricted (Nestle, F.O. et al., Nature Medicine 4 (1998) 328-332; Thurner, B. et al., J Exp Med 190 (1999) 1669-1678). However, there is increasing evidence that the efficacy and longevity of cytotoxic T cell responses against tumors can be increased by the involvement of MHC class II - restricted helper T cells. Hence, an improved vaccination method would foresee the
25   combinatorial use of MHC class II associated tumor peptides in addition to MHC class I antigens.

Knowledge of MHC class II-restricted cancer antigens recognized by CD4+ T helper cells lags behind the identification of class I-restricted antigens (Wang, R.-F., Trends in Immunol 22 (2001) 269-276). One reason is that transfection of cDNA libraries from
30   tumor cells into target cells and then using anti-tumor T cells to identify the appropriate transfectants and antigenic epitopes - a method successfully employed with MHC class I molecules - is not effective because the encoded proteins do not travel to the MHC class II pathway in APCs.

An innovative alternative is to use autologous DCs pulsed with tumor cells or
35   particular tumor marker proteins and sequence the peptides associated to MHC or Hsp

molecules on DCs. This approach, however, has not been employed so far, since DCs are non-dividing in vitro and only available in very small amounts from peripheral blood or bone marrow. Moreover, peptide purification and sequencing techniques were by far too insensitive, as yet, to directly identify disease-associated peptides by this or any other

5   approach.

The same limitations are evident in the context of autoimmune diseases, such as rheumatoid arthritis (RA). RA patients suffer from systemic destruction of their joint tissue, which is mediated by auto-aggressive T lymphocytes and auto-antibodies. The presence of both auto-reactive T cells and antibodies rely on the presentation of MHC

10   class II-restricted peptide antigen. In accordance with that, HLA-DR molecules, particularly the genes DRB1*0401 and DRB1*0404 in people of European descent, revealed to be major risk factors and confer increased susceptibility to RA (Marrack P et al. Nat. Med., 2002, 7: 899-905). Several candidate auto-antigens, such as collagen type II, fiaggrin, IgG and cartilage glycoprotein gp39, have been proposed. The corresponding

15   autoantigenic peptides have been elucidated by indirect means only, e.g. the capacity to activate T cell clones present in serum or synovial fluid in RA patients. Recently, it became clear that none of the major CD4+ T cell clones accumulating in inflamed synovia of joints of RA patients recognizes the respective epitopes (Kotzin BL et al., PNAS (2000), 97, 291-296). A likely rationale for this is that it was not possible, as yet, to sequence HLA-DR-

20   associated peptides from synovial tissue in RA, so that direct identification of autoantigenic peptides is still missing.

Hence, similar as in the tumor field, establishing a methodology that allows sequencing of naturally processed MHC and Hsp-associated peptides in the femtomolar range is a major need.

25

The present invention provides methods for isolating and identifying femtomolar amounts of peptide antigens presented by 0.1 to 5 µg MHC molecules or Hsp receptors isolated from an organism or from cells derived from an organism. Said methods concern

30   immune monitoring of diseases, e.g. autoimmune diseases, the design of individualized peptide vaccines for the treatment of diseases, e.g. of cancer and the quality control of vaccines e.g. those based on dendritic cells. The methods of the invention have the advantage that the identity of bound and/or presented antigenic peptides can be elucidated from very small amounts of bodily fluids or cells isolated from an mammalian organism.

The described methods ensure that the antigenic peptides isolated and identified are those that are bound and/or presented by peptide receptors in vivo or are those that are naturally-processed and presented by APCs, preferably DCs in vitro.

5

Fig. 1A is a diagram showing an overview of the methodology following strategy 1 (direct approach): MHC class II-peptide compelxes or Hsp-peptide complexes are isolated directly from tissue or bodily fluids thereby leading to the identification of naturally processed MHC class II or Hsp associated antigens presented in vivo.

10   Fig. 1B is a diagram showing an overview of the methodology following strategy 2 (indirect approach): Dendritic cells (DCs), the most specialized antigen presenting cells (APCs), are brought in contact with an antigenic source (e.g. bodily fluids) under optimal conditions for antigen uptake and antigen processing. As a control, DCs are cultured under the same conditions without contact with antigens. After maturation of DC antigen

15   loaded MHC class II molecules are purified and the respective MHC class II associated antigenic peptides are isolated and identified.

Fig. 2A illustrates strategy 2 and is a mass spectrometric analysis of HLA-DR bound peptides isolated from mature dendritic cells which were mock-treated (upper panel) or pulsed with the influenza vaccine Inflexal Berna V™, containing virosome-encapsulated

20   recombinant hemagglutinin from Influenza virus (lower panel). The three major signals induced by treatment with Inflexal Berna V™ are marked by arrows and numbers.

Fig. 2B shows the protein sequence (one-letter-code) of the influenza hemagglutinin protein from strain B / Yamanashi / 166 / 98. The newly identified HLA-DR epitope (cf. Fig. 2A) is underlined.

25   Fig. 3A contains a representative mass spectrometric analysis of the repertoire of HLA-DR bound peptides isolated from mature dendritic cells which have been mock treated (upper panel) or pulsed with the necrotic melanoma cell line UKRV-Mel-15a (lower panel). Marked is the peptide peak $(M+H^+)=1820.6$ which became dominant in the profile upon contact with melanoma cells.

30   Figs. 3B shows the corresponding MALDI-PSD fragmentation spectrum of the peptide with the experimental mass $(M+H+)=1820.6$. This peptide was induced by necrotic melanoma cells (Fig. 3A). Data base search led to the identification of the vimentin epitope vimentin(202-217) (cf. Table 2).

Figs. 3C shows an Ion trap MS-MS spectrum of a peptide with the experimental mass (M+H+)=1820.6. This peptide was induced by necrotic melanoma cells (Fig. 3A). Data base search led to the identification of the vimentin epitope vimentin(202-217) (cf. Table 2).

5

Method

The present invention provides a method for isolating disease-associated antigenic peptides in femtomolar amounts allowing their identification which method comprises providing complexes of peptide receptors with antigenic peptides from a mammalian

10   organism in an amount of 0.1 to 5 μg, preferably in an amount of 0.2 to 3 μg. This quantity equals to the amount of material which is normally available from biopsies or bodily fluids of patients or healthy donors. The lowest amount of material necessary in the prior art is about 200 μg MHC class II molecules derived from an unlimited source (inbred mice) (Dongre AR et al., EJI 2001, 31, 1485-94). This is about two orders of magnitude more

15   material than available from human patient material.

The amount of tissue or bodily fluid necessary to obtain e.g. 100 ng MHC class II molecules depends on the number of cells that do express MHC class II and on the expression rate of MHC class II molecules: e.g. 100 ng of MHC class II are equivalent to about 2×10^5 mature DCs or 5 to 10×10^6 peripheral blood monocytes or about 5×10^7

20   peripheral blood mononuclear cells which can be obtained from about 50 ml of blood.

The high sensitivity required for identifying MHC or Hsp-associated peptides is explained by the fact that each type of these peptide receptors, e.g. human MHC class II gene product HLA-DR1, carries about 500 to 1000 different antigenic peptides (Chicz RM et al., J Exp. Med. 1993, 178, 27-47; Chicz RM & Urban RG, Immunol. Today, 1993, 15:

25   155-160). However, most of the 500 to 1000 different peptides attain very low copy numbers and, therefore, are not very likely to play a physiological role. Especially in the MHC class II field, those peptides that are of immunological relevance e.g. those that activate helper T cells, attain moderate to high copy numbers (Latek RR & Unanue ER, Immunol. Rev. 1999, 172: 209-228). These peptides cover about 40 to 50% of the total

30   amount of peptide material eluted from MHC class II molecules and equal to about 10 to 200 individual peptides.

Many MHC class II associated peptides are represented as a set of 2 to 5 C- and N-terminal truncation variants (Rudensky AY et al, Nature 1992, 359, 429-431; Chicz et al. Nature 1992, 358: 764-768) sharing a common core sequence of about 10 to 13 amino

35   acids which is essential for recognition by the T cell receptor. These truncation /

elongation variants constitute the same T cell epitope. This means that the number of different epitopes, which are of importance is actually smaller, ranging from about 5 to 70 different epitopes. Thus, the abundance of immunologically relevant epitopes ranges from 0.2% to 5%.

5   In more detail, the method of the present invention comprises (a) providing complexes of peptide receptors with antigenic peptides isolated from a mammalian organism in an amount of 0.1 to 5 µg, and (b) eluting the associated antigenic peptides from the peptide receptors.

Origin of the peptides

10   The antigenic peptides of the present invention are peptides which are associated with peptide receptors from tissue or body fluids or cells of a mammalian organism or from antigen presenting cells derived from a mammalian organism. They may be bound to transmembrane peptide receptors comprising MHC I and MHC II molecules presenting the antigenic peptides at the cell surface to T cells of the immune system. The antigenic
15   peptides may also be bound to intra- or extracellular MHC molecules. Peptides may also be bound to intracellular peptide receptors relating to the heat shock protein (Hsp) family.

The antigenic peptides comprise self antigens, tumor antigens, autoantigens, viral, bacterial, and parasitic antigens. Some antigenic peptides may induce tolerance. Other antigenic peptides may elicit an immune response and are therefore immunogenic
20   peptides. The antigenic peptides of the present invention may be naturally processed antigenic peptides, that means they are generated from antigenic proteins by the proteolytic system of the respective cell and loaded onto peptide receptors. The antigenic peptides may also be non-naturally processed synthetic or recombinant antigenic peptides which may have been administered to an organism where they have been loaded onto
25   peptide receptors without further processing or they may have been contacted with cells expressing peptide receptors in cell culture or with isolated peptide receptors in vitro.

Therefore, the methods of the present invention comprise antigenic peptides, which are naturally-processed antigenic peptides, as well as antigenic peptides which are synthetic or recombinant antigenic peptides.

30   As all these peptide receptors are able to accommodate a broad variety of peptide ligands (see above), each single peptide whose sequence has to be determined is represented in only femtomolar amounts. 1 µg MHC class II (16 pmol) may carry dominant peptide species, with each single peptide attaining an occupancy of 0.1 - 2%, which equals to about 16 - 320 femtomoles. The methods of the present invention allow

the isolation of these femtomolar amounts of antigenic peptides from 0.1 to 5 µg of peptide receptors loaded with peptides and their subsequent sequencing.

Origin of the peptide receptors

5 In a further embodiment, the methods of the present invention relate to peptide receptors comprising all proteins binding antigenic peptides, comprising MHC class II molecules, MHC class I molecules and Hsp proteins.

Furthermore, complexes of peptide receptors with antigenic peptides may be isolated from diverse body fluids of an organism, e.g. of blood, serum, ascites, synovial fluid, from tissue samples of an organism, e.g. tumor biopsies, or from cells isolated from an
10 organism.

Complexes of peptide receptors with antigenic peptides may be isolated from a mammalian organism, preferably from a human organism.

The peptide receptors are isolated from an organism in an amount of 0.1 to 5 µg. Preferably, the peptide receptors are isolated from an organism in an amount of 0.2 to 3
15 µg.

Origin of the cellular material

The methods of the present invention encompass all cells expressing peptide receptors, e.g. all cells comprising Hsp molecules with associated antigens, all cells comprising MHC molecules with associated antigens; cells expressing MHC I or Hsp
20 molecules comprise nearly all nucleated cells; cells expressing MHC II molecules comprise B cells, macrophages, dendritic cells, monocytes, thymic epithelial cells, microglial cells, activated T cells and endothelial and epithelial cells after induction with pro-inflammatory cytokines e.g. IFNgamma. The cells expressing MHC II molecules are also referred to as antigen presenting cells (APCs) (Unanue, E.R.. Macrophages, antigen presenting cells and
25 the phenomena of antigen handling and presentation. In: Fundamental Immunology, 2nd edition (editor Paul, W.E) New York, Raven Press, 1989).

Solubilization of peptide receptors from cells

For the purification of peptide receptor-peptide complexes from cells or tissue, the membranes of the cells or tissue have to be solubilized. Cell lysis may be carried out with
30 methods known in the art, e.g. freeze-and-thaw cycles and the use of detergents, and combinations thereof. Preferred lysis methods are solubilization using detergents, preferably TX-100, NP40, n-octylglucoside, Zwittergent, Lubrol, CHAPS, most preferably TX-100 or Zwittergent 3-12. Cell debris and nuclei have to be removed from cell lysates

containing the solubilized receptor-peptide complexes by centrifugation. Therefore, in a further embodiment of the present invention, the complexes of peptide receptors with antigenic peptides are isolated from the cells with methods comprising solubilization with a detergent.

Nano-scale purification of MHC-peptide complexes

Furthermore, the invention provides the purification of the MHC-peptide complexes from cell lysates by methods comprising immunoprecipitation or immunoaffinity chromatography. For the immunoprecipitation or immunoaffinity chromatography, antibodies specific for MHC class I or MHC class II molecules and suitable for these methods are used. The specific antibodies are preferably monoclonal antibodies, and are covalently or non-covalently e.g. via Protein A, coupled to beads, e.g. sepharose or agarose beads. A selection of the broad panel of anti-HLA antibodies used in the prior art comprises:

anti-HLA-DR antibodies: L243, TU36, DA6.147, preferably L243; anti-HLA-DQ antibodies: SPVL3, TU22, TU169, preferably TU22 and TU169; anti-HLA-DP antibody B7/21 and anti-HLA-A,B,C antibodies W6/32 and B9.12.

Monoclonal antibodies specific for different MHC class I and MHC class II molecules may be commercially obtained (e.g. Pharmingen, Dianova) or purified from the supernatant of the respective hybridoma cells using Protein A- or Protein G- affinity chromatography. Purified monoclonal antibodies may be coupled by various methods known in the art, preferably by covalently coupling antibody amino groups to CNBr-activated sepharose.

Immunoisolation of MHC molecules may be performed by incubating the antibody-beads with the cell lysate under rotation for several hours or chromatographically by pumping the cell lysate through a micro-columns. Washing of the antibody-beads may be performed in eppendorf tubes or in the microcolumn. The efficacy of the immunoprecipitation may be analysed by SDS-PAGE and western blotting using antibodies recognizing denatured MHC molecules (anti-HLA-DRalpha: 1B5; anti-HLA class I: HC10 or HCA2).

Purification of Hsp-peptide complexes

Hsp-peptide complexes may be purified by methods known in the art (Binder, R. et al. J. Immunol., (2000), 165: 2582-2587). In brief, cells may be homogenized in a hypotonic buffer and fractionated by ammonium sulfate precipitation. The 50% precipitates may be applied to ADP-affinity beads and, subsequently, to DEAE anion

exchange beads in ordered to purify Hsp70-peptide complexes. The 80% precipitates of the above ammonium sulfate precipitation may be used to purify Hsp90 family protein-peptide complexes by a combination of Concanavalin A affinity chromatography and DEAE anion exchange chromatography.

5   Elution and fractionation of peptide receptor-associated peptides

By eluting the peptides from the receptor molecules, a complex mixture of naturally processed peptides derived from the source of potential antigen and from polypeptides of intra- or extracellular origin, is obtained. Only after elution, peptides can be fractionated and subjected to sequence analysis.

10   The antigenic peptides in the methods of the present invention may be eluted by a variety of methods known in the art, preferably by using diluted acid, e.g., diluted acetonitrile (Jardetzky TS et al., Nature 1991 353, 326-329), diluted acetic acid and heating (Rudensky AY et al., Nature 1991, 353, 622-626; Chicz RM et al., Nature 1992, 358, 764-768) or diluted trifluoro acetic acid at 37°C (Kropshofer H et al., J Exp Med 1992, 175,
15   1799-1803). Most preferably, the peptides are eluted at 37°C with diluted trifluoro acetic acid.

In a further embodiment, the sequestered peptide receptor-peptide complexes are washed with water or low salt buffer before elution in order to remove residual detergent contaminants. The low salt buffer may be a Tris, phosphate or acetate buffer in a
20   concentration range of 0.5 – 10 mM, preferably in a concentration of 0.5 mM. In a more preferred embodiment, the peptide receptor-peptide complexes are washed with ultrapure water (sequencing grade) conventionally used for HPLC analysis, preferably with ultrapure (sequencing grade) water from MERCK. The washing step may be carried out by ultrafiltration. The ultrafiltration may be carried out in an ultrafiltration tube with a cut-
25   off of 30 kD, 20 kD, 10 kD or 5 kD, preferably of 30 kD and a tube volume of 0.5 – 1.0 ml ("Ultrafree" tubes; Millipore). The washing in the ultrafiltration tube may be carried out 4 to 12 times, preferably 6 to 10 times, with a volume of 10 to 20 times the volume of the beads carrying the receptor-peptide complexes, preferably with a volume of 15 times the beads. The eluted peptides may be separated from the remaining peptide receptor
30   molecules using the same ultrafiltration tube. The eluted peptides may then be lyophilized.

Peptide sequence analysis by liquid chromatography-mass spectrometry (LC-MS)

In a further embodiment of the present invention, the isolated antigenic peptides are fractionated, sequenced and identified. By sequencing it is understood that the amino acid sequence of the individual peptides in the mixture of isolated antigenic peptides is
35   elucidated by methods adequate to sequence femtomolar amounts of peptides. By

identifying it is understood that it is established from which proteins or polypeptides the antigenic peptides are derived and which sequence they constitute within these proteins or polypeptides.

In a first step, the complex mixture of eluted peptides may be fractionated by one of a variety of possible chromatographic methods, e.g. by reversed phase, anion exchange, cation exchange chromatography or a combination thereof. Preferably, the separation is performed by C18-reverse phase chromatography or by reversed-phase / cation exchange two-dimensional HPLC, denoted as MudPit (Washburn MP et al., Nat Biotechnol., (2001), 19, 242-247).

The fractionation is done in a HPLC mode utilizing fused-silica micro-capillary columns which are either connected to a nano-flow electrospray source of a mass spectrometer or to a micro-fractionation device which spots the fractions onto a plate for MALDI analysis.

A variety of mass spectrometric techniques are suitable, preferably MALDI-post source decay (PSD) MS or electrospray ionization tandem mass spectrometry (ESI-MS), most preferably ion-trap ESI-MS.

The sequences of the individual peptides can be determined by means known in the art. Preferably, sequence analysis is performed by fragmentation of the peptides and computer-assisted interpretation of the fragment spectra using algorithms, e.g. MASCOT or SEQUEST. Both computer algorithms use protein and nucleotide sequence databases to perform cross-correlation analyses of experimental and theoretically generated tandem mass spectra. This allows automated high through-put sequence analysis.

Qualitative peptide analysis by MALDI mass spectrometry

For qualitative analysis of the whole peptide repertoire obtained upon elution, matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry may be carried out. Using settings that do not fragment the peptides, MALDI-TOF analysis provides a rough overview with regard to the complexity of the peptide mixture and the presence of dominant peptides.

Quantitative peptide analysis

To estimate the quantity of single peptides eluted from peptide receptors, the run through of the micro-capillary column may be analyzed by a flow-through UV detector operated at a detection wave-length of 214 nm. For quantitation the peak areas of peptides

to be analyzed are compared with peak areas of graded amounts of synthetic standard peptides.

Strategy 1 (ex-vivo approach)

Strategy 1 of the present invention is used to isolate antigenic peptides which were loaded onto peptide receptors inside an organism (ex vivo approach, Fig. 1A).

The present invention relates to a method for isolating and identifying MHC or Hsp associated peptides in femtomolar amounts which method comprises providing 0.1 to 5 µg MHC-peptide or Hsp-peptide complexes from a mammalian organism. This amount of peptide receptors equals to the amount of material which is normally available from biopsies or bodily fluids of patients or healthy donors, e.g. 100 ng of MHC class II-peptide complexes can be purified from about 5×10^7 peripheral blood mononuclear cells isolated from about 50 ml of blood.

The MHC-peptide or Hsp-peptide complexes may be purified from isolated cells e.g. blood monocytes, from a mixture of cells e.g. peripheral blood mononuclear cells, from tissue, e.g. tumor biopsies, or from body fluids e.g. ascites or synovial fluid.

The body fluids may contain MHC-peptide or Hsp-peptide complexes bound to cells present in the body fluid, e.g. in synovial fluid, bound to vesicles present in the body fluid e.g. apoptotic vesicles or exosomes derived from cells (Denzer K et al., J Cell Science, 2000, 113, 3365-3374), or MHC-peptide complexes may be present in soluble form, due to shedding from the plasma membrane, e.g. soluble MHC class I and MHC class II molecules (Aultman D et al., Human Immunol., 1999, 60, 239-244).

MHC-peptide or Hsp-peptide complexes may be isolated from diverse body fluids of an organism, e.g. of blood, serum, ascites, synovial fluid or from tissue samples of an organism, e.g. biopsies, excised primary or secondary tumors or from cells isolated from an organisms.

Complexes of peptide receptors with antigenic peptides may be isolated from cells, tissue or body fluids from a mammalian organism, preferably from a human organism.

30

Strategy 2 (in vitro approach)

Strategy 2 of the present invention foresees isolation of antigenic peptides which have been loaded onto peptide receptors outside an organism, e.g. in cell culture (in vitro approach, Fig. 1B).

In a further embodiment the present invention relates to a method for isolating antigenic peptides in femtomolar amounts which method comprises (a) providing MHC expressing cells in a numer providing 0.1 to 5 µg MHC molecules, (b) contacting the cells with a source of potential antigen, (c) isolating MHC molecule-antigenic peptide complexes from the cells and (d) eluting the associated peptides from the MHC molecules.

The MHC expressing cells may be MHC I or MHC II expressing cells (APCs). Preferably, APCs are dendritic cells, more preferably, the APCs are immature dendritic cells, most preferably, the APCs are immature dendritic cells generated from peripheral blood monocytes.

Dendritic cells may be generated from peripheral blood monocytes or from bone marrow-derived CD34+ stem cell-precursors. The peripheral blood mononuclear cells (PBMCs) may be isolated from blood samples by density gradient centrifugation. The monocytes may then be isolated from PBMCs by methods known in the art, e.g. by sorting with magnetic beads. The source of dendritic cells may be mammalian species, preferably humans. The monocytes may then be differentiated in cell culture to become immature dendritic cells. The differentiation state may be monitored by flow-cytometric analysis, e.g. using upregulation cell surface markers CD83, CD80, CD86, HLA-DR.

The amount of tissue or bodily fluid necessary to obtain e.g. 100 ng MHC class II molecules depends on the number of cells that do express MHC class II and on the expression rate of MHC class II molecules: e.g. 100 ng of MHC class II are equivalent to about 2×10^5 mature DCs or 5 to 10×10^6 peripheral blood monocytes or about 5×10^7 peripheral blood mononuclear cells which can be obtained from about 50 ml of blood.

The APCs are then contacted with a source of potential antigen. The APCs, preferably the immature dendritic cells, are at the same time triggered to mature by methods known in the art, e.g. incubation with inflammatory cytokines, like TNF alpha or a mixture of TNF alpha, IL-6, IL1beta PGE2.

The source of potential antigen offered to the APCs may be selected from the group comprising tumor tissue, tumor cells, tumor cell lines, gene-modified tumor cell lines, a crude cellular lysate of these cells or cell lines, tumor cell-derived heat shock proteins, pathogens, known viral, bacterial and parasitic antigens, tissues subject to immune attack,

known self antigens, autoantigens, body fluids or tissue biopsies from patients with tumor, autoimmune or infectious diseases, body fluids or tissue biopsies from healthy individuals as reference controls. Control APCs are treated equivalently except that they are not exposed to a source of potential antigen. The source of potential antigen may be derived
5   from different mammalian species, preferably from human.

The APCs may be contacted with a source of potential antigen which is taken up by the APCs by receptor-mediated uptake or by fluid phase uptake and internalized. The cells may also be infected with a source of potential antigen, e.g., with a virus.

By eluting the peptides from the MHC molecules, a set of naturally processed
10   peptides derived from the source of potential antigen as well as from polypeptides of intracellular or extracellular origin, is obtained.

Epitope validation for MHC-associated peptides

The peptide sequences identified by the methods of the invention may be validated
15   by one of several criteria, comprising MHC binding motif, MHC binding capacity and T cell recognition.

MHC binding motifs are common structural characteristics of peptides associated to a particular MHC molecule (allelic variant) which are necessary to form stable complexes with MHC molecules. Peptide ligands eluted from MHC class I molecules are relatively
20   short, ranging from 8 to 11 amino acids. Moreover, 2 or 3 side chains of the peptide are relevant for binding. The position of the respective amino acid side chains varies with the HLA allele, most often two of these so-called "anchor" residues are located at positions 2 and 9. With respect to a particular anchor position, only 1 or 2 amino acids normally can function as anchor amino acids e.g. leucine or valine V at position 2 in the case of HLA-
25   A2.

In the case of MHC class II molecules, the peptide length varies from 12 to 18 amino acids and even longer peptides can bind since both ends of the peptide binding groove are open. Most HLA class II molecules accommodate up to 4 anchor residues at relative positions P1, P4, P6 and P9 contained in a nonameric core region. This core region,
30   however, can have variable distance from the N-terminus of the peptide. In the majority of cases, 2-4 N-terminal residues precede the core region. Hence, the P1 anchor residues is located at positions 3, 4 or 5 in most HLA class II associated peptides. Peptides eluted from HLA-DR class II molecules share a big hydrophobic P1 anchor, represented by tyrosine, phenylalanine, tryptophane, methionine, leucine, isoleucine or valine.

The position and the exact type of anchor residues constitute the peptide binding motif which is known for most of the frequently occurring HLA class II allelic products. A computer algorithm allowing motif validation in peptide sequences is "Tepitope", available by vaccinome (www.vaccinome.com).

5   The MHC binding capacity of the peptides identified by the methods of the present invention may be tested by methods known in the art using, for example, isolated MHC class II molecules and synthetic peptides with amino acid sequences identical to those identified by the method of the invention (Kropshofer H et al., J. Exp. Med. 1992; 175, 1799-1803; Vogt AB et al., J. Immunol. 1994; 153, 1665-1673; Sloan VS et al., Nature 1995;

10   375, 802-806). Alternatively, a cellular binding assay using MHC class II expressing cell lines and biotinylated peptides can be used to verify the identified epitope (Arndt SO et al., EMBO J., 2000; 19, 1241-1251)

In both assays, the relative binding capacity of a peptide is measured by determining the concentration necessary to reduce binding of a labelled reporter peptide by 50%

15   ($IC50$). Peptide binding with a reasonable affinity to the relevant HLA class II molecules attain IC50 values not exceeding 10-fold the IC50 of established reference peptides.

The same binding assays can also be used to test the ability of peptides to bind to alternative class II MHC molecules, i.e., class II MHC molecules other than those from which they were eluted using the method of the invention.

20   T cell recognition may represent another epitope verification procedure involving testing of peptides identified by the methods of the invention for their ability to activate CD4+ or CD8+ T cell populations. CD4+ T cell are activated by peptides binding to MHC class II molecules while CD8+ T cells are activated by peptides binding to MHC class I molecules. Peptides with amino acid sequences either identical to those identified by the

25   methods of the invention or corresponding to a core sequence derived from a nested group of peptides identified by the methods of the invention are synthesized. The synthetic peptides are then tested for their ability to activate CD4+ (or CD8+) T cells from (a) test subjects expressing the MHC class II (or MHC class I) molecule of interest and having at least one symptom of the disease; and (b) control subjects expressing the MHC class II (or

30   MHC class I) molecule of interest and having no symptoms of the disease. Additional control subjects can be those with symptoms of the disease and not expressing the MHC class II (or MHC class I) molecule of interest.

In some diseases (e.g., those with an autoimmune component) responsiveness in the CD4+ T cells of test subjects but not in CD4+ T cells of the control subjects described in

35   (b) provides confirmatory evidence that the relevant peptide is an epitope that activates

CD4+ T cells that can initiate, promote, or exacerbate the relevant disease. In other diseases (e.g., cancer or infectious diseases without an autoimmune component), a similar pattern of responsiveness and non-responsiveness to that described in the previous sentence would indicate that the relevant peptide is an epitope that activates CD4+ T cells
5   that can mediate immunity to the disease or, at least, a decrease in the symptoms of the disease.

CD4+ (or CD8+) T cell responses can be measured by a variety of in vitro methods known in the art. For example, whole peripheral blood mononuclear cells (PBMC) can be cultured with and without a candidate synthetic peptide and their proliferative responses
10   measured by, e.g., incorporation of [3H]-thymidine into their DNA. That the proliferating T cells are CD4+ (or CD8+) T cells can be tested by either eliminating CD4+ (or CD8+) T cells from the PBMC prior to assay or by adding inhibitory antibodies that bind to the CD4+ (or CD8+) molecule on the T cells, thereby inhibiting proliferation of the latter. In both cases, the proliferative response will be inhibited only if CD4+ (or CD8+) T cells are
15   the proliferating cells. Alternatively, CD4+ (or CD8+) T cells can be purified from PBMC and tested for proliferative responses to the peptides in the presence of APC expressing the appropriate MHC class II (or MHC class I) molecule. Such APCs can be B-lymphocytes, monocytes, macrophages, or dendritic cells, or whole PBMC. APCs can also be immortalized cell lines derived from B-lymphocytes, monocytes, macrophages, or
20   dendritic cells. The APCs can endogenously express the MHC class II (or MHC class I) molecule of interest or they can express transfected polynucleotides encoding such molecules. In all cases the APCs can, prior to the assay, be rendered non-proliferative by treatment with, e.g., ionizing radiation or mitomycin-C.

As an alternative to measuring cell proliferation, cytokine production by the CD4+ T
25   cells can be measured by procedures known to those in art. Cytokines include, without limitation, interleukin-2 (IL-2), interferon-gamma (IFN-gamma), interleukin-4 (IL-4), TNF-alpha, interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12) or TGF-beta. Assays to measure them include, without limitation, ELISA, and bio-assays in which cells responsive to the relevant cytokine are tested for responsiveness (e.g., proliferation) in
30   the presence of a test sample.

Alternatively, cytokine production by CD4+ lymphocytes can be directly visualized by intracellular immunofluorescence staining and flow cytometry.

35

Applications

The methods of the present invention can be applied to identify peptides involved in the pathogenesis of a wide range of diseases, especially those in which susceptibility has been associated with expression of one or several particular MHC alleles or those where MHC-restricted T cell responses are lacking.

Candidate diseases include, without limitation, autoimmune diseases (e.g. rheumatoid arthritis (RA), type I diabetes, multiple sclerosis (MS), coeliac disease, myasthenia gravis (MG) and systemic lupus erythematosus (SLE)), cancer (e.g. melanoma, breast cancer, B cell lymphomas, prostate cancer, renal cancer) or infectious diseases (e.g. diseases caused by HIV, hepatitis C virus, measles virus, mycobacteria).

One aspect of the invention is a therapeutic purpose, wherein one or more of the identified peptides are used to vaccinate patients against cancer or infectious diseases. To this end, the relevant peptides may be directly administered to the patient, in an amount sufficient for the peptides to bind to the MHC molecules, and provoke activation of T cells followed by T cell-mediated lysis of infected or cancer cells.

Alternatively, the relevant peptides may be utilized for the generation of vaccines based on DCs. In this case, autologous DCs derived from patients' monocytes may be pulsed with the relevant peptides or recombinant proteins containing the relevant peptide sequences. Particularly, in vaccination against tumors, a combination of MHC class I- and class II-associated tumor antigenic peptides may be used to pulse DCs. Similarly, nucleic acid molecules which encode the relevant peptides may be incorporated into a vector in order to transfect tumor cells. These transfected tumor cells may be fused with DCs. In any of these cases, DCs presenting the relevant peptides in context of the appropriate MHC molecules will be administered to a patient for triggering a T cell response.

A further therapeutic application of the peptides relates to the situation where identified peptides are autoantigens in the context of autoimmune diseases. In this case, complexes of the respective autoantigenic peptide and its restricting MHC class II molecules may be targeted by chimeric or humanized antibodies. This approach may lead to diminishment of autoantigenic MHC class II peptide complexes and, thus, to a decline in the number of autoaggressive CD4+ T helper cells which belong to one of the driving forces in autoimmunity.

Therefore, the present invention provides the use of the described methods for the design of individualized peptide vaccines for the treatment of diseases, preferably cancer.

Beyond that, the method of the invention can be exploited for several diagnostic purposes. First, peptides of the invention may be used as response markers to track the efficacy of a therapeutic regime. Essentially, one can determine the baseline value for the relevant peptide, e.g. an autoantigenic peptide in the synovial fluid of rheumatoid arthritis

5   patients, using strategy 1 of the invention, administer a given therapeutic drug and then monitor levels of the autoantigenic peptide thereafter, observing changes in peptide levels as indicia of the efficacy of the regime.

Therefore, the present invention provides the use of the described methods for the control of the efficacy of a therapeutic treatment.

10   In the same manner MHC-associated peptides derived from blood of patients suffering from autoimmune diseases (e.g. RA, type I diabetes, MS, coeliac disease, MG or SLE) may be used as response markers for therapeutic drugs against autoimmune diseases.

Likewise, autoantigenic peptides which are only found in certain stages or phases of an autoimmune disease may be utilized as stage-specific markers.

15   Therefore, the present invention provides the use of the described methods for immune monitoring of diseases, preferably of autoimmune diseases.

A further aspect of the invention is a method for controlling the quality of vaccines based on DCs. Autologous DCs used as vaccines against tumors are subjected to tumor antigens in order to load MHC molecules of DCs with appropriate tumor antigenic

20   peptides. A prerequisite of a high-quality DC vaccine is a high copy number of tumor antigenic peptides bound to the relevant MHC molecules. Applying strategy 2 of this invention, the presence or absence of the relevant peptides can be tested prior to utilizing the respective DC preparations for vaccination. Similarly, the quality of vaccines based on other APCs, e.g. macrophages or B cells, could be determined.

25   Therefore, the present invention provides the use of the described methods for the quality control of vaccines.

Finally, the method of the invention not only allows the identification of MHC- or Hsp-associated peptides, but, at the same time, the protein wherefrom the peptide is derived. These proteins may be used as diagnostic markers in the very same manner as

30   described above for the corresponding peptides.

In a further embodiment, the present invention provides a method of producing a pharmaceutical composition comprising the steps of the methods of the present invention,

producing the identified peptides and optionally modifying them and formulating the product obtained with a pharmaceutically acceptable carrier or diluent.

Depending on the intended use of the composition, adequate carriers or diluents have to be added. Examples of such carriers and methods of formulation for

5   pharmaceutical compositions may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the identified substance.

While it is possible for the antigenic peptide to be administered in a pure or substantially pure form, it is preferable to present it as a pharmaceutical composition,

10   formulation or preparation.

The pharmaceutical compositions of the present invention, both for veterinary and for human use, comprise an antigenic peptide as described above, together with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other

15   ingredients of the formulation and not deleterious to the recipient thereof. The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any method well-known in the pharmaceutical art.

All methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the

20   formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired formulation.

Formulations suitable for intravenous, intramuscular, subcutaneous, or intraperitoneal administration conveniently comprise sterile aqueous solutions of the

25   active ingredient with solutions which are preferably isotonic with the blood of the recipient. Such formulations may be conveniently prepared by dissolving solid active ingredient in water containing physiologically compatible substances such as sodium chloride (e.g. 0.1-2.0M), glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering said solution

30   sterile. These may be present in unit or multi-dose containers, for example, sealed ampoules or vials.

The formulations of the present invention may incorporate a stabilizer. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids which may be used either on their own or as admixtures. These stabilizers are

35   preferably incorporated in an amount of about 0.11 about 10,000 parts by weight per part

by weight of immunogen. If two or more stabilizers are to be used, their total amount is preferably within the range specified above. These stabilizers are used in aqueous solutions at the appropriate concentration and pH. The specific osmotic pressure of such aqueous solutions is generally in the range of about 0.1 to about 3.0 osmoles, preferably in the

5   range of about 0.8 to about 1.2. The pH of the aqueous solution is adjusted to be within . the range of about 5.0 to about 9.0, preferably within the range of 6-8. In formulating the antigenic peptide of the present invention, anti-adsorption agent may be used.

Having now generally described this invention, the same will become better

10   understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Examples

The examples below are in connection with the figures described above and based on the methodology summarized in Figure 1A and Figure 1B and described in detail in the following. Commercially available reagents referred to in the examples were used

5   according to manufacturer's instructions unless otherwise indicated.

Methodology of the invention

Cell lines and culture

The study was performed with human dendritic cells which were differentiated from

10   monocytes, as described below. Monocytes were purified from human peripheral blood. In addition, the melanoma cell lines UKRV-Mel-15a and UKRV-Mel-20c (Eichmueller S et al., Exp Dermatol 2002; 11, 292-31) were utilized.

All cells were cultured in RPMI 1640 medium (short: RPMI) supplemented with 1 mM Pyruvat, 2 mM Glutamine and 10% heat-inactivated fetal calf serum (Gibco BRL,

15   Rockville, MD).

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was obtained from the local blood bank as standard buffy coat preparations from healthy donors. Heparin (200 I.U./ml blood, Liquemine, Roche) was

20   used to prevent clotting. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in LSM® (1.077-1.080g/ml; ICN, Aurora, OH) at 800g (room temperature) for 30 min. PBMCs were collected from the interphase and washed twice in RPMI containing 20 mM Hepes (500g for 15 min, 300g for 5 min). In order to remove erythrocytes, PBMCs were treated with ALT buffer (140 mM ammonium chloride, 20 mM

25   Tris, pH 7.2) for 3 min at 37°C. PBMCs were washed twice with RPMI containing 20 mM Hepes (200g for 5 min).

Generation of dendritic cells from peripheral blood monocytes.

Monocytes were isolated from PBMCs by positive sorting using anti-CD14 magnetic

30   beads (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. Monocytes were cultured in RPMI supplemented with 1% non-essential amino acids

(Gibco, BRL, Rockville, MD), 50 ng/ml recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF; S.A. 1.1×10^7U/mg) (Leucomax; Novartis, Basel Switzerland) and3 ng/ml recombinant human IL-4 (S.A. 2.9×10^4U/mg) (R&D Systems, Minneapolis, MN). Monocytes were seeded at 0.3 x 10^6/ml in 6-well plates (Costar) for 5 days to obtain immature dendritic cells.

The quality of monocyte-derived immature dendritic cells was routinely monitored by flow-cytometric analysis conforming to the phenotype: CD1a (high), CD3 (neg.), CD14 (low), CD19 (neg.), CD56 (neg.), CD80 (low), CD83 (neg.), CD86 (low) and HLA-DR (high). In contrast, mature dendritic cells (cf. below) display the following phenotype: CD1a (low), CD80 (high), CD83 (high), CD86 (high) and HLA-DR (high). Monoclonal antibodies against CD1a, CD3, CD14, CD19, CD56, CD80, CD83, CD86 as well as the respective isotype controls were purchased from Pharmingen (San Diego, CA).

Exposure of dendritic cells to necrotic melanoma cells

Melanoma cells lines were rendered necrotic by 4 cycles of freezing in liquid nitrogen and subsequent thawing at room temperature. The percentage of necrotic cells was monitored by light microscopy. To feed dendritic cells with melanoma cell-derived antigen, 6 x 10^6 immature dendritic cells were exposed to 1.8 x 10^7 necrotic cells (3:1 ratio). At the same time maturation of dendritic cells was induced by adding 10 ng/ml recombinant human tumor necrosis factor (TNFalpha; S.A. 1.1×10^5U/mg). As a control, 6 x 10^6 dendritic cells were incubated with TNFalpha alone.

After 24-48 hrs of co-culture, mature dendritic cells were harvested by centrifugation at 300g for 10 min. Cells were washed with RPMI containing 10% FCS and transferred to an eppendorf tube. After centrifugation at 400g for 3 min, the supernatant was completely removed and the cells were frozen at -70°C.

Generation of anti-HLA class II beads

The anti-HLA-DR monoclonal antibody (mAb) L243 (ATCC, Manassas, VA) was produced by culturing the respective mouse hybridoma cell line. mAb L243 was purified using ProteinA sepharose (Pharmacia, Uppsala, Sweden) and immobilized to CNBr-activated sepharose beads (Pharmacia) at a final concentration of 2.5 mg/ml, according to the manufacturer's protocol. L243 beads were stored in PBS containing 0.1% Zwittergent 3-12 (Calbiochem, La Jolla, CA).

Nano-scale purification of HLA-DR-peptide complexes

Pellets of frozen dendritic cells were resuspended in 10-fold volume of ice cold lysis buffer (1% Triton-X-100, 20 mM Tris, pH 7.8, 5 mM $MgCl_2$, containing protease inhibitors chymostatin, pepstatin, PMSF and leupeptin (Roche, Mannheim, Germany))
5   and lysed in a horizontal shaker at 1000 rpm, 4°C for 1h. The cell lysate was cleared from cell debris and nuclei by centrifugation at 2000g, 4°C for 10 min. The lysate was co-incubated with L243 beads (5-10 μl L243 beads per 100 μl cell lysate) in a horizontal shaker at 1000 rpm, 4°C for 2 hrs. Immunoprecipitated HLA-DR-peptide complexes bound to L243 beads were sedimented by centrifugation at 2000g, 4°C for 5 min and washed three
10   times with 300 μl 0.1% Zwittergent 3-12 (Calbiochem) in PBS.

The efficacy of depletion of HLA-DR-peptide complexes was monitored by analyzing the respective cell lysates before and after immunoprecipitation. In parallel, aliquots of the beads were analyzed by western blotting using the anti-HLA-DRa-specific mAb 1B5 (Adams, T.E. et al., Immunology 50 (1983) 613-624).

15

Elution of HLA-DR-associated peptides

HLA-DR-peptide complexes bound to L243 beads were resuspended in 400 μl H_2O (HPLC-grade; Merck, Darmstadt, Germany), transferred to an ultrafiltration tube, Ultrafree MC, 30 kD cut-off (Millipore, Bedford, MA) and washed 10 times with 400 μl
20   H_2O (HPLC-grade) by centrifugation for 2-4 min at 14000 rpm at 4°C. For eluting the bound peptides, 50 μl 0.1% trifluoracetic acid (Fluka, Buchs, Switzerland) in H_2O (HPLC-grade) was added and incubation was performed for 30 min at 37°C. Eluted peptides were collected in a new eppendorf tube by centrigugation of the Ultrafree unit at 14000 rpm for 3 min at RT and immediately lyophilized in a Speed-Vac™ vacuum centrifuge.

25

Fractionation of peptides by nano-HPLC

Lyophilized peptides eluted from HLA-DR molecules were resolved in 0.05% trifluoroacetic acid, 5% acetonitrile (Merck, Darmstadt, Germany) in H_2O, (HPLC-grade) and separated on a 75 μm x 15 cm C18 PepMap capillary (C18; 3μm; 100 Å) (LC-Packings,
30   Amsterdam, Netherlands) connected to a FAMOS™ autosampler and an ULTIMATE™ nano-flow HPLC (Dionex, Olten, Switzerland). The following non-linear gradient at a constant flow rate of 200 nl/min was used: 0-40min 5-50% system B; 40-50 min 50-90% system B. System A was 0.05% trifluoroacetic, 5% acetonitrile/H_2O and system B was

0.04% trifluoroacetic, 80% acetonitrile/H₂O. The separation was monitored via dual UV absorption at 214 nm and 280 nm. Fractions (400 nl) were collected using the fraction collector PROBOT™ (BAI, Weiterstadt, Germany) and spotted onto an AnchorChip 600/384 MALDI-MS target (Bruker, Bremen, Germany).

5

Sequence analysis of peptides by mass spectrometry

MALDI-TOF mass spectrometry

Peptides spotted onto an AnchorChip plate were co-cristallized with matrix (10 mg/ml; a-cyano-4-hydroxy-cinnamic acid (Merck, Darmstadt, Germany), 50%
10 acetonitrile, 0.1% trifluoroacetic acid). For qualitative analysis of the whole peptide repertoire, samples were analyzed on an Ultraflex™ MALDI-TOF mass spectrometer (Bruker, Bremen, Germany), according to the manufacturer's protocol.

Ion Trap MS/MS mass spectrometry

15 To perform high-throughput sequencing of complex peptide mixtures, the MudPIT (multidimensional protein identification technology) was used (Washburn MP et al., Nat Biotechnol 19 (2001), 242-247) which is based on a liquid chromatographic fractionation followed by mass spectrometric sequencing.

To this end, the lyophilized peptides eluted from HLA molecules were resuspended
20 in a buffer containing 5% (v/v) acetonitrile, 0.5% (v/v) acetic acid, 0.012% (v/v) heptafluoro butyric acid (HFBA) and 5% (v/v) formic acid. The sample was separated on a fused-silica microcapillary column (100 μm i.d. × 365 μm) generated by a Model P-2000 laser puller (Sutter Instrument Co., Novato, CA). The microcolumn was packed with 3 μm / C18 reverse-phase material (C18-ACE 3 μm [ProntoSIL 120-3-C18 ACE-EPS, Leonberg,
25 Germany]) followed by 3 cm of 5 μm cation exchange material (Partisphere SCX;Whatman, Clifton, NJ).

A fully automated 8-step gradient separation on an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) was carried out, using the following buffers: 5% ACN/0.02% HFBA/0.5% acetic acid (buffer A), 80% ACN/0.02% HFBA/0.5% acetic
30 acid (buffer B), 250 mM ammonium acetate/5% ACN/0.02% HFBA/0.5% acetic acid (buffer C), and 1.5 M ammonium acetate/5% ACN/0.02% HFBA/0.5% acetic acid (buffer D). The first step of 106 min consisted of a 100 min gradient from 0 to 80% buffer B and a 6 min hold at 80% buffer B. The next 6 steps (106 min each) are characterized by the

following profile: 5 min of 100% buffer A, 2 min of x% buffer C, 5 min of 100% buffer A, a 3 min gradient from 0 to 10% buffer B, a 55 min gradient from 10 to 35% buffer B, a 20 min gradient from 35 to 50% buffer B, a 16 min gradient from 50 to 80% buffer B. The 2 min buffer C percentages (x) in steps 2-7 were as follows: 10, 20, 30, 40, 70, 90, and 100%.

5 Step 8 consisted of the following profile: a 5 min 100% buffer A wash, a 20 min salt wash . with 100% buffer D and a 100 min gradient from 0-80% buffer B.

The HPLC column was directly coupled to a Finnigan LCQ ion trap mass spectrometer (Finnigan, Bremen, Germany) equipped with a nano-LC electrospray ionization source. Mass spectrometry in the MS-MS mode was performed according to the

10 manufacturer's protocol. The identification of peptides was done by the sequest algorithm against the swiss.fasta database.

MALDI-PSD mass spectrometry

As an alternative to doing sequence analysis by ion trap MS/MS, as described above,

15 MALDI-PSD analysis was performed on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker, Bremen, Germany) using the software FLEXControl 1.1 Alpha for data acquisition. Calibration was achieved by using a tryptic digest of human serum albumin (Merck, Darmstadt, Germany). Peptide mixtures were first scanned in a reflectron mode. Peptides of interest were then selected for lift mode (MALDI-PSD analysis). The peptide

20 fragmentation spectra obtained were automatically evaluated using the Xmas 5.1.2 and Biotools 2.1 Software (Bruker) and used for sequence identification in a non-redundant protein database using the MASCOT algorithm (http://www.matrixscience.com).

Example 1

25 Strategy 1 (Fig. 1A) was used to identify peptides associated to HLA-DR molecules expressed on the surface of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation. From 50 ml blood 5.3 x 10^7 PBMCs were recovered. The cell types typically present in PBMCs are T lymphocytes (about 50%), B lymphocytes (5-10%), monocytes (15-25%) and natural killer

30 cells (about 6%). Peripheral blood dendritic cells are also present but only in very low amounts (< 0.5%). Analysis of PBMCs by flow cytometry revealed that both B cells and monocytes express considerable amounts of HLA-DR molecules, while natural killer cells and T cells stain negative. The small amount of dendritic cells in PBMCs cannot be

visualized by FACS. Human T cells are able to up-regulate HLA-DR upon activation, however, activated T cells are normally absent from peripheral blood.

Although the number of B cells present in PBMCs is 2 to 3-fold lower compared to monocytes, their HLA-DR expression level is about 2-fold higher. This means that in
5 lysates from PBMCs the number of HLA-DR molecules originating from B cells is comparable to the number of HLA-DR molecules from monocytes.

PBMCs were lysed in TX-100 and HLA-DR molecules were precipitated using anti-DR mAb L243. Precipitation was controlled by western blot analysis using anti-DRalpha mAb 1B5. Quantitative western blot analysis using purified HLA-DR molecules as a
10 reference revealed that about 200 ng HLA-DR was purified from 5.3×10^7 cells. HLA-DR associated peptides were eluted in 0.1% TFA and the peptide mixture was fractionated using 2-dimensional cation-exchange/ reversed phase liquid chromatography (MudPit). Sequencing was done by high-throughput ion trap mass spectrometry and data base search was performed using human data bank "humangp". The peptides identified with a cross-
15 correlation > 2.0 are listed in Table 1.

27 peptides could be identified: 8 peptides were derived from human serum albumin and constituted a nested set of peptides typical for MHC class II associated peptides (N- and C-terminal elongation / truncation variants of the same epitope); 3 peptides were derived from apolipoprotein AII, again representing only one epitope; 3 peptides were
20 derived from alpha1 anti-trypsin and represented one epitope; 4 peptides from protein disulfide isomerase related protein ERp72 (1 epitope). The last 9 peptides were derived from different proteins and, thus, represented 9 different epitopes.

15 peptides (4 epitopes) were derived from extracellular proteins that are major constituents of human serum: serum albumin is the most abundant protein in serum,
25 apolipoprotein AII is a constituent of high density lipoproteins (HDLs), alpha 1 anti-trypsin is a well known serum protease inhibitor. Ferritin light chain (donor of peptide nr. 10) is present in virtually all cells and at low concentrations in plasma. Most likely, these proteins were internalized by fluid phase uptake, and after proteolytic processing the respective peptides were loaded onto HLA-DR molecules inside the APC (monocyte or B
30 cell). Alternatively, these proteins or fragments of the respective proteins could have bound to cell surface HLA-DR molecules.

The lysosomal-associated multi-transmembrane protein (lam5) is the donor of peptide nr. 4 and is expressed in haematopoietic cells. The subcellular localization of lam 5 is the lysosome, so it is already present in the loading compartment of HLA-DR molecules,
35 where it can bind before or after proteolytic cleavage. HLA class I molecules (giving rise to

peptide nr. 19) are present in nearly all nucleated cells, thus it is likely to be derived from the APC itself. Alternatively, HLA class I can be taken up from the serum where shedded HLA class I molecules have been described.

PDI ERp72 is an ER resident protein that has been described to be expressed in muscle and lung but also in a lymphoblastoid cell line. ERp72 gave rise to peptides nr. 20 to 23. Pyruvate kinase is a cytosolic protein and exists in several isoforms with isoform M1 being expressed in muscle, heart and brain, and isoform M2 is described for fetal tissue. The epitope (peptides 20-23) is present in both isoforms. Peptide nr. 24 is derived from actin alpha 1, a cytosolic protein that is highly expressed in skeletal muscle. Thus, all three epitopes could be derived from muscle cells that might have released their cellular proteins into the serum due to micro-tissue injury or damage.

Peptide nr. 5 is derived from F-box helicase 1, however, little is known, as yet, about the tissue expression of this protein.

Peptides nr. 25, 26, 27 are derived from proteins that could be allocated to chromosomes 17, 6 and 4, respectively, but there was no information about the respective proteins or their function, tissue expression or subcellular localization.

The bovine analogue (TPTLVEVSRSLGKVGTR) of the serum albumin epitope (peptides nr. 11-18) has been described as a HLA-DR-associated epitope in context of the DR alleles DRB1*1101/ DRB1*1104 (Verreck FA et al., Immunogenetics 1996; 43, 392-397). It has been identified by Edman sequencing of self-peptides derived from cultured EBV-transformed B cells. The peptide binding motif of both of these alleles requires an aromatic or aliphatic residue at position P1, an aliphatic residue at P4 and a basic residue at P6 (Verreck FA et al., Immunogenetics 1996; 43, 392-397). These requirements are also fulfilled in the human serum albumin epitope derived from PBMCs and identified here (P1 = L, P4 = V, P6 = R), (peptides 11-18), but also in peptides 1-4, 6-10, 20-23 and 27 (Table 1). Moreover, an epitope overlapping with the alpha1 anti-trypsin peptide found here has been described in EBV B cells in the context of DRB1*0402 (Friede T et al., 1996, Biochim Biophys Acta 1996; 1316, 85-101).

The DR allele coexpressed with DRB1*1101 is DRB3*0202, which has the following requirements for peptide binding: aromatic or aliphatic residue at position P1, an asparagine at P4 and a polar residue at P6 (Verreck FA et al., Immunogenetics 1996; 43, 392-397). These requirements are fulfilled by peptides 5 and 19. Thus, most of the identified peptides (n=21) contain the same motif, indicating that they are derived from the same HLA-DR allotype or from more than on HLA-DR allotype sharing a common motif.

Example 2

Strategy 2 (Fig. 1B) was used to identify novel peptides associated to HLA-DR molecules expressed on the surface of dendritic cells that are exposed to a potential antigen. In this case, the antigenic source was a commercially available vaccine against
5 influenza virus, denoted as INFLEXAL Berna V (Berna, Bern, Switzerland).

Dendritic cells were differentiated from peripheral blood monocytes and cultured in a concentration of 0.5 x 10^6 cells/ml. 6 x 10^6 dendritic cells were exposed to the vaccine INFLEXAL Berna V (Berna, Bern, Switzerland) for 24 hrs by adding INFLEXAL Berna V at a concentration of 100 µl/ml (corresponding to 3 µg/ml hemagglutinin derived from
10 influenza virus). At the same time maturation of dendritic cells was induced by adding TNFalpha (10 ng/ml). As a control, the same amount of dendritic cells (6 x 10^6) were cultured in the absence of the antigen, but in then presence of TNFalpha (10 ng/ml).

Both sets of dendritic cells were lysed in detergent TX-100 and HLA-DR molecules were precipitated by using anti-DR mAb L243 immobilized to sepharose beads. HLA-DR
15 associated peptides were eluted with 0.1% TFA and analyzed by MALDI-MS (Fig. 2A): The upper panel shows the complex mixture of HLA-DR-associated self-peptides from unpulsed DCs. The 3 dominant peptides displaying a mass-to-charge ratio of m/z = 2334, m/z = 2545 and m/z = 2676, correspond to variants of CLIP, the class II associated invariant chain peptide (Riberdy JM et al., Nature 1992; 360, 474-477), a dominant self
20 peptide in mature dendritic cells. The lower panel shows the peptide repertoire of DCs that were pulsed with INFLEXAL. Comparison of both MS spectra revealed that 3 novel signals became dominant in the MALDI-MS peptide profile of DCs upon contact with the INFLEXAL vaccine. These 3 novel signals appeared at m/z = 1969.4, m/z = 2097.6 and m/z = 2196.6 (Fig. 2A).

25 The hemagglutinin proteins contained in INFLEXAL Berna V are derived from 3 different influenza strains: strain A / New Caledonia / 20 / 99; strain A / Panama / 2007 / 99 ; strain B / Yamanashi / 166 / 98 (according to the recommendation of the WHO taking into account the genetic diversity among the circulating viruses (Lindstrom SE et al., J. Virol. 1999, 73, 4413-4426)).

30 A search for the identified masses in the three different hemagglutinin sequences of the above mentioned influenza strains revealed that all three peptides represented length variants of one epitope located in influenza hemagglutinin, strain B / Yamanashi / 166 / 98 (Fig.2B).

This epitope HA(253-271) KPGKTKTIVYQRGILLPQ contains the MHC peptide binding motif for DRB1*0101 and DRB5*0101 using I-260 as P1, Q-264 as P4 and L269 as P9 anchor residue (anchor residues are underlined).

MHC peptide binding studies using the synthetic peptide with the amino acid
5 sequence KPGKTKTIVYQRGILLPQ confirmed the binding capacity to alleles DRB1*0101 and DRB5*0101 and revealed the same for DRB1*0401. Thus, the newly identified epitope derived from influenza hemagglutinin (strain B / Yamanashi / 166 / 98) reveals an epitope with promiscuous binding capacity.

10   Example 3

Furthermore, strategy 2 (Fig. 1B) was used to identify novel HLA-DR-associated tumor peptides. Thus, dendritic cells were exposed to a necrotic melanoma cell line, UKRV-Mel-15a.

3 x 10^6 cells dendritic cells were co-incubated with 9 x 10^6 necrotic cells of the
15 melanoma line UKRV-Mel-15a. and cultured for 24 hrs in presence of TNFalpha (10 ng/ml). As a control, 3 x 10^6 cells dendritic cells were cultured in presence TNFalpha (10 ng/ml) only.

Both sets of dendritic cells were lysed in detergent TX-100 and HLA-DR molecules were precipitated using anti-DR mAb L243. HLA-DR associated peptides were eluted with
20 0.1% TFA and analyzed by MALDI-MS (Fig. 3A):

In this example, the HLA-DR associated peptides from both DC cultures were compared by MALDI-MS spectrometry and only the peptide signals contained in the profile of DCs pulsed with melanoma cells were used to identify new epitopes by successive sequencing.

25   MALDI-MS analysis revealed one dominant signal with an observed mass of m/z = 1820.6 in the spectrum of pulsed DCs as compared to unpulsed DCs (Fig. 3A).

Sequencing by MALDI-PSD fragmentation resulted in a novel epitope derived from the tumor antigen vimentin: vimentin(202-217) with the amino acid sequence TLQSFRQDVDNASLA (Fig. 3B). Sequence analysis by ion trap MS-MS confirmed this
30 sequence (Fig. 3C).

Vimentin(202-217) and several other known melanoma antigens share a common motif suitable for binding to HLA-DR4 B1*0401 molecules (Table 2). In contrast to the

typical DRB1*0401 peptide binding motif derived from self- and foreign antigenic peptides, the peptides derived from melanA, CDC27, tyrosinase and vimentin display asparartic acid (D) instead of threonine (T) or serine (S) at anchor position P6. The relevance of this peculiarity remains to be investigated.

5   Vimentin is known to be a marker protein in a variety of benign and malign tumors. Together with melanA/MART-1, tyrosinase and S100, vimentin is routinely used to trace melanoma cells in clinical specimens from melanoma patients. Interestingly, melanoma clones with low invasive potential have a high vimentin expression, whereas vimentin is downregulated in highly invasive melanoma cell clones (Gutgemann A et al., Arch

10 Dermatol Research 2001; 293, 283-290). In contrast, enhanced expression of vimentin is observed in poorly differentiated and metastatic prostate carcinoma (Lang SH et al., Prostate 2002; 52, 253-263). Moreover, vimentin is overexpressed in human renal cell carcinoma in relation to normal renal tissue (Stassar MJ et al. Br. J. Cancer 2001; 85, 1372-1382). Likewise, >95% of tumor cells in classical Hodgkin's lymphoma are vimentin

15 positive, whereas T-cell-rich B-cell lymphomas are negative for vimentin (Rudiger T et al., Am J Surg Path 1998, 22, 1184-91).

     Vimentin(202-217) peptide identified by the method of the invention is the first vimentin derived HLA class II restricted epitope described so far.

20   **Example 4**

     In this example strategy 2 (Fig. 1B) was used to identify as many peptides as possible which were bound to HLA-DR molecules of dendritic cells (DCs) after TNFalpha-induced maturation and exposure to potential antigens. Sequencing was done by high-throughput ion trap MS/MS technology.

25   Thus, 5 x 10^6 cells dendritic cells were co-incubated with 1.5 x 10^7 necrotic cells of the melanoma line UKRV-Mel-20c and cultured for 24 hrs in presence of TNFalpha (10 ng/ml). As a control, 5 x 10^6 cells dendritic cells were cultured in presence TNFalpha (10 ng/ml) only.

     Both sets of dendritic cells were lysed in detergent TX-100 and HLA-DR molecules

30 were precipitated using anti-DR mAb L243. HLA-DR associated peptides were eluted with 0.1% TFA and analyzed by LC-high-throughput ion trap MS/MS technology.

The peptide sequences identified from unpulsed DCs (control) are given in Table 3, whereas the peptide sequences identified from DCs pulsed with necrotic melanoma cells are listed in Table 4.

5   35 individual peptide sequences from HLA-DR molecules of DCs were identified in the absence of melanoma cells, and 40 peptide sequences were found in the presence of UKRV-Mel20c melanoma cells. Comparison of the peptide sequences revealed that 21 peptides are identical (nr. 1-21), 14 sequences (11 epitopes) are specific for unpulsed DCs and 17 sequences (9 epitopes) are only presented after melanoma cell pulse.

10   7 of the 9 peptides induced by melanoma cells share the binding motif of DRB1*0405 (Table 5). Importantly, 3 of the 9 melanoma cell induced epitopes are derived from known tumor marker proteins, namely translation factor IF-4A1, translation factor EF-1alpha and interferon-gamma (IFNgamma)-inducible P78.

    The translation initiation factor IF-4A1 is consistently overexpressed in melanoma cell lines in relation to normal human melanocytes. IF-4A1 overexpression seems to be an 15 important feature of melanoma cells and might contribute to their malignant transformation (Eberle J et al., Int. J. Cancer 1997; 71, 396-401).

    A wide evidence suggests the involvement of ribosomal elongation factors (EF) at the onset of oncogenesis. Altered expression of EF-1alpha, a core component of protein synthesis, has been linked to transformed phenotypes in several studies. Overexpression of 20 EF-1alpha mRNA has been correlated with increased metastatic potential in mammary adenocarcinoma and EF-1alpha has a considerable degree of homology with the prostate oncogene PTI-1 (Gopalkrishnan RV et al., Int J Biochem Cell Biol 1999; 31, 151-162; Edmonds BT et al., J Cell Sci 1996; 109, 2705-2714).

    A naturally processed self-peptide derived from EF-1alpha containing the same 25 epitope as peptides nr. 42 and 43 (Table 4) has also been eluted from HLA-DR molecules purified from EBV transformed B cell lines (Verreck FA et al., Immunogenetics 1996; 43, 392-397).

    Prostate cancer progression from an hormon-dependent to an hormon-independent state includes a cascade of genetic alterations caused by activation of oncogenes and/ or 30 inactivation of tumor suppressor genes. Several genes were identified which are highly overexpressed in androgen-independent cancer cell lines. Amongst others, the interferon-inducible genes 1-8U and p78 were identified (Markku H et al., Lab Invest. 2000; 80, 1259-1268.).

Thus, the peptides derived from translation factor IF-4A1, translation factor EF-1alpha and IFNgamma-inducible P78 identified by the method of the invention are new candidate tumor antigens that can be used as diagnostic markers or therapeutic vaccines.

TABLE 1

HLA-DR bound peptides derived from peripheral blood mononuclear cells

PEPTIDE Nr.	LENGTH	OBSERVED MASS	SEQUENCE[a]	PROTEIN SOURCE
1	16	1756.4	SKEQLTPLIKKAGTEL	apolipoprotein AII
2	15	1643.6	SKEQLTPLIKKAGTE	apolipoprotein AII
3	14	1556.8	KEQLTPLIKKAGTE	apolipoprotein AII
4	16	1765.6	SVLLFIEHSVEVAHGK	lysosomal-associated multi-transmembrane protein lam5
5	17	1795.8	VDGILSNCGIEKESDLC	F-box DNA helicase 1
6	17	1888.2	GTQGKIVDLVKELDRDT	alpha1 anti-trypsin
7	16	1830.8	TQGKIVDLVKELDRDT	alpha1 anti-trypsin
8	14	1614.5	TQGKIVDLVKELDR	alpha1 anti-trypsin
9	14	1623.6	GKNIKIISKIENHE	pyruvate kinase M1/M2
10	14	1655.5	LDEEVKLIKKMGDH	ferritin light chain
11	22	2353.9	TPTLVEVSRNLGKVGSKCCKPH	serum albumin
12	18	1872.5	STPTLVEVSRNLGKVGSK	serum albumin
13	17	1785.6	TPTLVEVSRNLGKVGSK	serum albumin
14	17	1756.8	VSTPTLVEVSRNLGKVG	serum albumin
15	17	1744.3	STPTLVEVSRNLGKVGS	serum albumin
16	16	1657.4	STPTLVEVSRNLGKVG	serum albumin
17	15	1569.6	TPTLVEVSRNLGKVG	serum albumin
18	14	1513.4	TPTLVEVSRNLGKV	serum albumin
19	16	1852.5	GKDYIALNEDLRSWTA	HLA class I heavy chain
20	16	1794.9	GYPTIKILKKGQAVDY	PDI ERp72
21	15	1737.9	YPTIKILKKGQAVDY	PDI ERp72
22	15	1631.1	GYPTIKILKKGQAVD	PDI ERp72

23	14	1574.8	YPT<u>IKILKKGQA</u>VD	PDI ERp72
24	16	1791.1	SY<u>ELPDGQVITIGN</u>ER	actin alpha 1
25	14	1624.6	SV<u>ILKILPSYQ</u>EPH	*(chr 17)*
26	14	1466.8	AK<u>IHIDIVLVGGS</u>TR	*(chr 6)*
27	15	1716.1	NAL<u>LVRTKKVPQ</u>VS	*(chr 4)*

[a]The 9-mer core region containing the putative peptide binding motif of each peptide is underlined

TABLE 2

HLA-DR4 (B1*0401)-associated melanoma antigens

PEPTIDE Nr.	LENGTH	SEQUENCE [a]	POSTITION	PROTEIN SOURCE	REF.
54	15	RNG<u>Y</u>RAL<u>M</u>DKSLHVG	51-65	Melan-A	b
55	15	MNF<u>S</u>WAM<u>D</u>LDFKGAN	768-772	CDC27	b
56	13	S<u>Y</u>LQ<u>D</u>SDPDSFQD	448-462	Tyrosinase	b
53	16	TLQS<u>F</u>RQ<u>D</u>VDNASLAR	202-217	Vimentin	this study

[a] The sequences of the peptides are aligned according to the peptide binding motif of HLA-DR4

(DRB1*0401): P1 anchor: W,Y,F; P4 anchor: D,E,L. The peculiarity of "D" (instead of T,S or N) at anchor

position 6 is marked in bold.

[b] R.-F. Wang, Trends in Immunology 22, 269-276 (2001)

TABLE 3

HLA-DR bound peptides derived from mature dendritic cells

PEP. Nr.	LENGTH	OBSERVED MASS	SEQUENCE[a]	PROTEIN SOURCE	CELLULAR COMPARTMENT
1	17	1829.0	FPEPIKLDNKNDRAKAS	Rab-7	cytosol
2	16	1746.9	HTGALYRIGDLQAFQG	CD98	surface
3	14	1552.8	TGALYRIGDLQAFQ	CD98	surface
4	16	1720.0	AKNQVAMNPTNTVFDA	HSC 70	cytosol
5	17	1786.0	NVLRIINEPTAAAIAYG	HSP 70	cytosol
6	18	1899.1	LNVLRIINEPTAAAIAYG	HSP 70	cytosol
7	14	1586.9	IDKVISTITNNIQQ	TGF-induc. Ig	surface
8	14	1667.8	DDVILNEPSADAPA	Integr. MP 2B	surface
9	15	1632.9	NSNQIKILGNQGSFL	CD4	surface
10	16	1642.0	NKEGLELLKTAIGKAG	α-Enolase	cytosol
11	15	1769.9	KVVVYLQKLDTAYDD	Cathepsin C	endosome
12	16	1883.0	KKVVVYLQKLDTAYDD	Cathepsin C	endosome
13	16	1898.0	KVVVYLQKLDTAYDDL	Cathepsin C	endosome
14	15	1746.9	YPRISVNNVLPVFDN	Cathepsin D	endosome
15	16	1800.9	TTAFQYIIDNKGIDSD	Cathepsin S	endosome
16	17	1871.9	TTAFQYIIDNKGIDSDA	Cathepsin S	endosome
17	16	1812.9	LPGQLKPFETLLSQNQ	GSH-S-Transf.	cytosol
18	17	1870.0	LPGQLKPFETLLSQNQG	GSH-S-Transf.	cytosol
19	16	1830.9	VSNEIVRFPTDQITPD	Myeloperoxid	endosome
20	17	1886.0	VDEVTIVNILTNRSNAQ	Annexin-II	cytosol
21	17	1897.4	TDGKDYIALNEDLSSWT	HLA-B	surface

22	14	1600.9	LAV<u>V</u>KSIRSIPYLA	Annexin-V	cytosol
23	15	1714.1	LLAV<u>V</u>KSIRSIPYLA	Annexin-V	cytosol
24	13	1614.9	VADK<u>I</u>QLINMLDK	PG-Kinase	cytosol
25	14	1717.8	DQV<u>I</u>KVFNDMKVRK	Cofilin	cytosol
26	15	1680.9	LRT<u>I</u>DVFDGNSGKMM	GAP-DH	cytosol
27	15	1628.9	GKVD<u>I</u>VAINDPFIDL	GAP-DH	cytosol
28	14	1576.8	DD<u>I</u>RGIQSLYGDPK	Metallo-Elastase	extracell.
29	15	1647.8	ADD<u>I</u>RGIQSLYGDPK	Metallo-Elastase	extracell.
30	15	1600.9	SSNV<u>V</u>HLIKNAYNKL	Integrin-β	surface
31	16	1756.9	LNQE<u>L</u>RADGTVNQIEG	Apolipoprotein D	extracell.
32	15	1611.8	GPLKF<u>L</u>HQDIDSGQG	Man-6-P-Rez.	surface
33	17	1880.9	GPLKF<u>L</u>HQDIDSGQGIR	Metallo-Elastase	cytosol
34	17	1906.9	VKSE<u>I</u>IPMFSNLASDEQ	Prot.-Phosphat.2A	cytosol
35	16	1821.8	GSHS<u>M</u>RYFHTAMSRPG	HLA-B	surface

[a]The putative P1 anchor of the HLA-DR bound peptide is underlined

TABLE 4

HLA-DR bound peptides derived from mature dendritic cells pulsed with the melanoma cell line UKRV-Mel-20c

PEPTI DE Nr.	LENGTH	OBSERVED MASS	SEQUENCE[a]	PROTEIN SOURCE	CELLULAR COMPARTMENT
1	17	1829.0	FPEPIKLDNKNDRAKAS	Rab-7	cytosol
2	16	1746.9	HTGALYRIGDLQAFQG	CD98	surface
3	14	1552.8	TGALYRIGDLQAFQ	CD98	surface
4	16	1720.0	AKNQVAMNPTNTVFDA	HSC 70	cytosol
5	17	1786.0	NVLRIINEPTAAAIAYG	HSP 70	cytosol
6	18	1899.1	LNVLRIINEPTAAAIAYG	HSP 70	cytosol
7	14	1586.9	IDKVISTITNNIQQ	TGF-induc. Ig	membrane
8	16	1667.8	DDVILNEPSADAPA	Integr. MP 2B	membrane
9	15	1632.9	NSNQIKILGNQGSFL	CD4	surface
10	16	1642.0	NKEGLELLKTAIGKAG	α-Enolase	cytosol
11	15	1769.9	KVVVYLQKLDTAYDD	Cathepsin C	endosome
12	16	1883.0	KKVVVYLQKLDTAYDD	Cathepsin C	endosome
13	16	1898.0	KVVVYLQKLDTAYDDL	Cathepsin C	endosome
14	16	1746.9	YPRISVNNVLPVFDN	Cathepsin D	endosome
15	16	1800.9	TTAFQYIIDNKGIDSD	Cathepsin S	endosome
16	17	1871.9	TTAFQYIIDNKGIDSDA	Cathepsin S	endosome
17	16	1812.9	LPGQLKPFETLLSQNQ	GSH-S-Transf.	cytosol
18	17	1870.0	LPGQLKPFETLLSQNQG	GSH-S-Transf.	cytosol
19	16	1830.9	VSNEIVRFPTDQITPD	Myeloperoxid	endosome

20	17	1886.0	VDEVT<u>I</u>VNILTNRSNAQ	Annexin-II	cytosol
21	17	1897.4	DGKD<u>Y</u>IALNEDLSSWTA	HLA-B	surface
36	16	1879.1	KRKT<u>V</u>TAMDVVYALKR	Histon H4	nucleus
37	16	1723.0	KRKT<u>V</u>TAMDVVYALK	Histon H4	nucleus
38	14	1950.1	AKRKT<u>V</u>TAMDVVYALKR	Histon H4	nucleus
39	14	1784.9	SPK<u>Y</u>IKMFVLDEADE	Transl. IF-4A1	cytosol
40	15	1915.9	SPK<u>Y</u>IKMFVLDEADEM	Transl. IF-4A1	cytosol
41	18	1886.1	GSSR<u>V</u>LITTDLLARGIDV	Transl. IF-4A1	cytosol
42	16	1576.8	TAQV<u>I</u>ILNHPGQISAG	Transl. EF-1α	cytosol
43	13	1647.8	V<u>I</u>ILNHPGQISAG	Transl. EF-1α	cytosol
44	17	1600.9	VYK<u>V</u>LKQVHPDTGISSK	Histon H2B	nucleus
45	14	1756.9	K<u>V</u>LKQVHPDTGISS	Histon H2B	nucleus
46	15	1611.8	K<u>V</u>LKQVHPDTGISSK	Histon H2B	nucleus
47	14	1730.9	KSK<u>I</u>EDIRAEQERE	IFN-γ induc. P78	cytosol
48	13	1601.9	KSK<u>I</u>EDIRAEQER	IFN-γ induc. P78	cytosol
49	16	1761.9	HNSL<u>I</u>ASILDPYSNAF	Mannose-Rec.	surface
50	15	1831.9	TTAYF<u>L</u>YQQQGRLDK	Inv. Chain	endosome
51	17	1908.2	NRQ<u>V</u>NKKLNKTDLPKLL	K Channel	surface
52	17	1930.0	AEF<u>L</u>LHMLKNAESNAEL	Ribosomal L17	cytosol

^aThe putative P1 anchor of the HLA-DR bound peptide is underlined

TABLE 5

HLA-DR bound peptides induced by melanoma cell line UKRV-Mel-20c and sharing the binding motif of DRB1*0405

PEPTIDE Nr.	LENGTH	OBSERVED MASS	SEQUENCE[a]	PROTEIN SOURCE
39	15	1784.9	SPKYIKMFVLDEADE	Transl. Factor eIF-4A
40	16	1915.9	SPKYIKMFVLDEADEM	Transl. Factor eIF-4A
47	14	1730.9	KSKIEDIRAEQERE	IFN-induc. P78
48	13	1601.9	KSKIEDIRAEQER	IFN-induc. P78
50	15	1831.9	TTAYFLYQQQGRLDK	Invariant Chain
51	17	1908.2	NRQVNKKLNKTDLPKLL	K - Channel
52	17	1930.0	AEFLLHMLKNAESNAEL	Ribos.Prot. L17

[a] The sequences of the peptides are aligned according to the peptide binding motif of HLA-DR4

5   (DRB1*0405): P1 anchor: Y,F,I,L,V; P4 anchor: M,I,V; P9 anchor: E,D
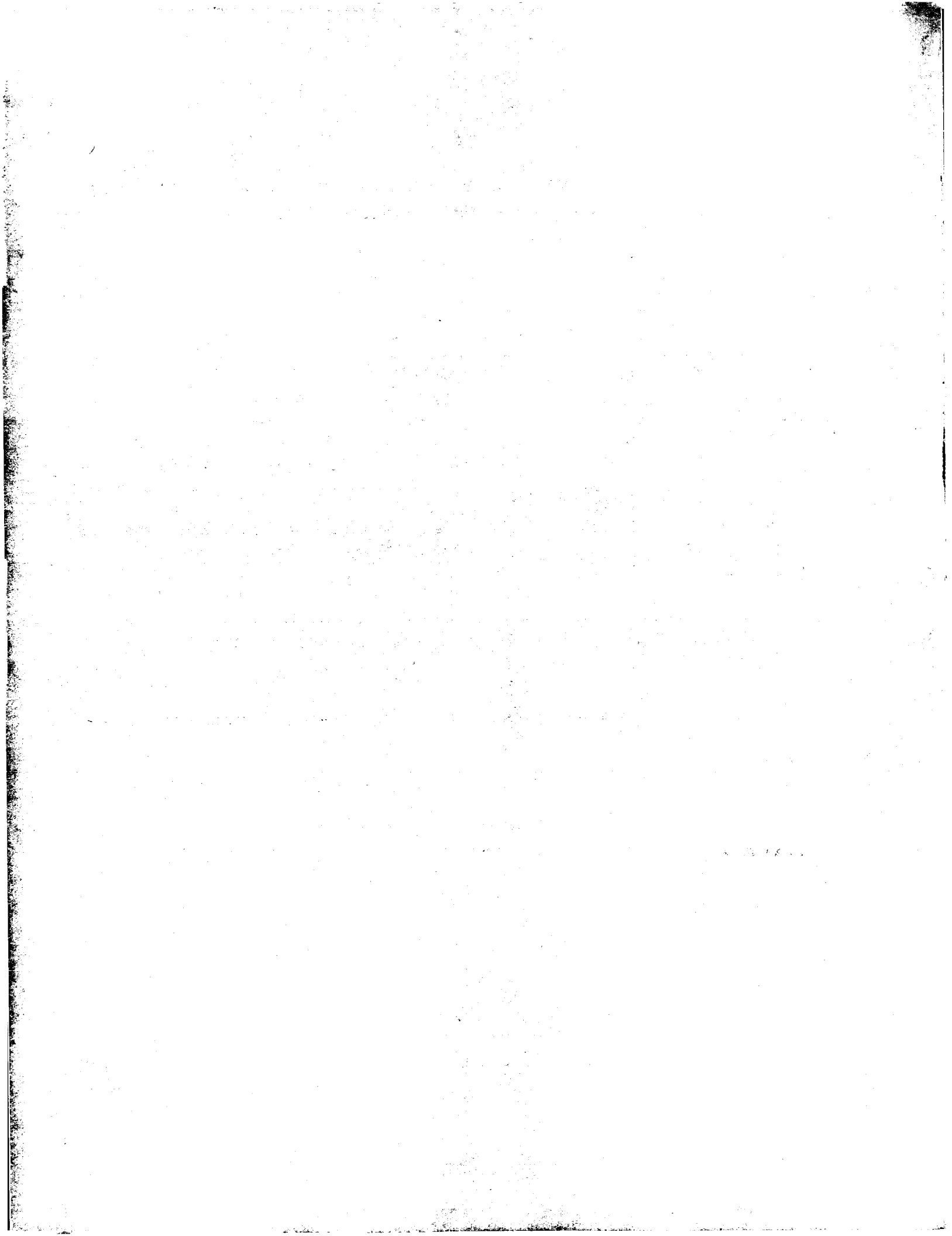
Claims

1. A method for isolating antigenic peptides in femtomolar amounts, which method comprises

(a) providing complexes of peptide receptors with antigenic peptides isolated from a mammalian organism in an amount of 0.1 to 5 µg and

(b) eluting the associated antigenic peptides from the peptide receptors.

2. The method according to claim 1, which method comprises isolating the complexes of peptide receptors with antigenic peptides in an amount of 0.1 to 5 µg from cells isolated from a mammalian organism.

3. The method according to claim 2, wherein the complexes of peptide receptors with antigenic peptides are isolated from the cells with methods comprising solubilization of the cells with a detergent and sequestration of the complexes of peptide receptors with antigenic peptides by immunoprecipitation or immunoaffinity chromatography.

4. The method according to claims 2 and 3, wherein the cells isolated from a mammalian organism are dendritic cells.

5. The method according to claims 1 to 4, wherein the sequestered complexes of peptide receptors with antigenic peptides are washed with water in an ultrafiltration tube before eluting the peptides.

6. The method according to claims 1 to 5, wherein the antigenic peptides are eluted from the peptide receptors using diluted acid.

7. The method according to claims 1 to 6, wherein the isolated antigenic peptides are fractionated, sequenced and identified.

8. The method according to claim 7, wherein the isolated antigenic peptides are fractionated, sequenced and identified by methods comprising liquid chromatography and mass spectrometry.

9. The method according to claims 1 to 8, wherein the antigenic peptides are naturally-processed antigenic peptides or non-naturally processed antigenic peptides administered to the organism.

10. The method according to claims 1 to 9, wherein the peptide receptors comprise Hsp molecules, MHC I molecules and MHC II molecules.

11. The method according to claims 2 to 10, wherein the cells are cells expressing peptide receptors belonging to the group comprising MHC I, MHC II and Hsp molecules.

12. The method according to claims 1 to 11, wherein the mammalian organism is a human organism.

13. A method for isolating antigenic peptides in femtomolar amounts, which method comprises

(a) providing complexes of peptide receptors with antigenic peptides isolated from cells, tissue or body fluid of a mammalian organism in an amount of 0.1 to 5 µg;

(b) washing the sequestered complexes of peptide receptors with antigenic peptides with water in an ultrafiltration tube;

(c) eluting the associated antigenic peptides from the peptide receptors at 37°C with diluted trifluoro acetic acid,

(d) sequencing and identifying the isolated peptides by liquid chromatography and mass spectrometry.

14. A method for isolating antigenic peptides in femtomolar amounts, which method comprises

(a) providing MHC expressing cells in a numer providing 0.1 to 5 µg MHC molecules

(b) contacting the cells of (a) with a source of potential antigen

(c) isolating MHC molecule-antigenic peptide complexes from the cells and

(d) eluting the associated peptides from the MHC molecules.

15. The method according to claim 14, wherein the MHC expressing cells are MHC I expressing cells.

16. The method according to claim 14, wherein the MHC expressing cells are MHC II expressing cells.

17. The method according to claim 16, wherein the MHC II expressing cells are dendritic cells.

18. The method according to claim 17, wherein the dendritic cells are exposed to a potential source of antigen as immature dendritic cells at the same time as they are induced to mature to dendritic cells.

19. The method according to claims 14 to 18, wherein the source of potential antigen belongs to the group comprising tumor cells, tumor cell lines, pathogens, viral, bacterial and parasitic antigens, autoantigens, body fluids e.g. serum, synovial fluid, ascites.

20. The method according to claims 14 and 19, wherein the complexes of peptide receptors with antigenic peptides are isolated from the cells with methods comprising solubilization of the cells with a detergent and sequestration of the complexes of peptide receptors with antigenic peptides by immunoprecipitation or immunoaffinity

5 chromatography.

21. The method according to claims 14 to 20, wherein the sequestered complexes of peptide receptors with antigenic peptides are washed with water in an ultrafiltration tube before eluting the peptides.

22. The method according to claims 14 to 21, wherein the antigenic peptides are

10 eluted from the peptide receptors using diluted acid.

23. The method according to claims 14 to 22, wherein the isolated antigenic peptides are fractionated, sequenced and identified.

24. The method according to claim 23, wherein the isolated antigenic peptides are fractionated, sequenced and identified by methods comprising liquid chromatography and

15 mass spectrometry.

25. The method according to claims 23 to 24, wherein the antigenic peptides derived from the source of potential antigen are identified by comparing the peptides identified from cells which have been contacted with a source of potential antigen with those which have been identified from cells which have not been contacted with a source of potential

20 antigen.

26. The method according to claims 14 to 25, wherein the antigenic peptides are naturally-processed antigenic peptides.

27. A method for isolating antigenic peptides in femtomolar amounts, which method comprises

25 (a) providing immature dendritic cells in a numer providing 0.1 to 5 µg MHC II molecules;
(b) contacting the cells of (a) with a source of potential antigen and inducing maturation of dendritic cells by adding TNFalpha;
(c) isolating MHC II molecule-antigenic peptide complexes from the cells with methods

30 comprising solubilization of the cells with the detergent TX-100 and sequestration of the complexes of MHC II molecules with antigenic peptides by immunoprecipitation or immunoaffinity chromatography;
(d) washing the sequestered complexes of MHC II molecules with antigenic peptides with water in an ultrafiltration tube;

(e) eluting the associated antigenic peptides from the MHC II molecules at 37°C with diluted trifluoro acetic acid, and

(f) sequencing and identifying the isolated peptides by liquid chromatography and mass spectrometry.

5   28. Use of the methods of claims 14 to 27 for the quality control of vaccines.

29. Use of the methods of claims 1 to 27 for immune monitoring of diseases.

30. Use of the methods of claims 1 to 27 for the control of the efficacy of a therapeutic treatment.

31. Use of the methods of claims 1 to 27 for the design of individualized peptide

10   vaccines for the treatment of diseases.

32. A method of producing a pharmaceutical composition comprising the steps of claims 7 to 13 or claims 23 to 27, producing the identified peptides and optionally modifying them and formulating the product obtained with a pharmaceutically acceptable carrier or diluent.

15   33. The method substantially as herein before described especially with reference to the foregoing Examples.

***

Abstract

The present invention relates to methods useful for isolating antigenic peptides from a limited quantity of cells or bodily fluid from a mammalian organism in an amount sufficient to determine their sequence and identity. Therefore this invention relates to methods for identifying novel disease-associated antigens, e.g. tumor antigens and antigens involved in autoimmune diseases, to be utilized for diagnostic or therapeutic purposes. The methods of the present invention can also be utilized for controlling the quality of vaccines. More specifically, the methods of the invention can be used for determining the sequence of antigenic peptides presented via peptide receptors of dendritic cells which are the most important antigen presenting cells of the body and valuable tools for vaccination.
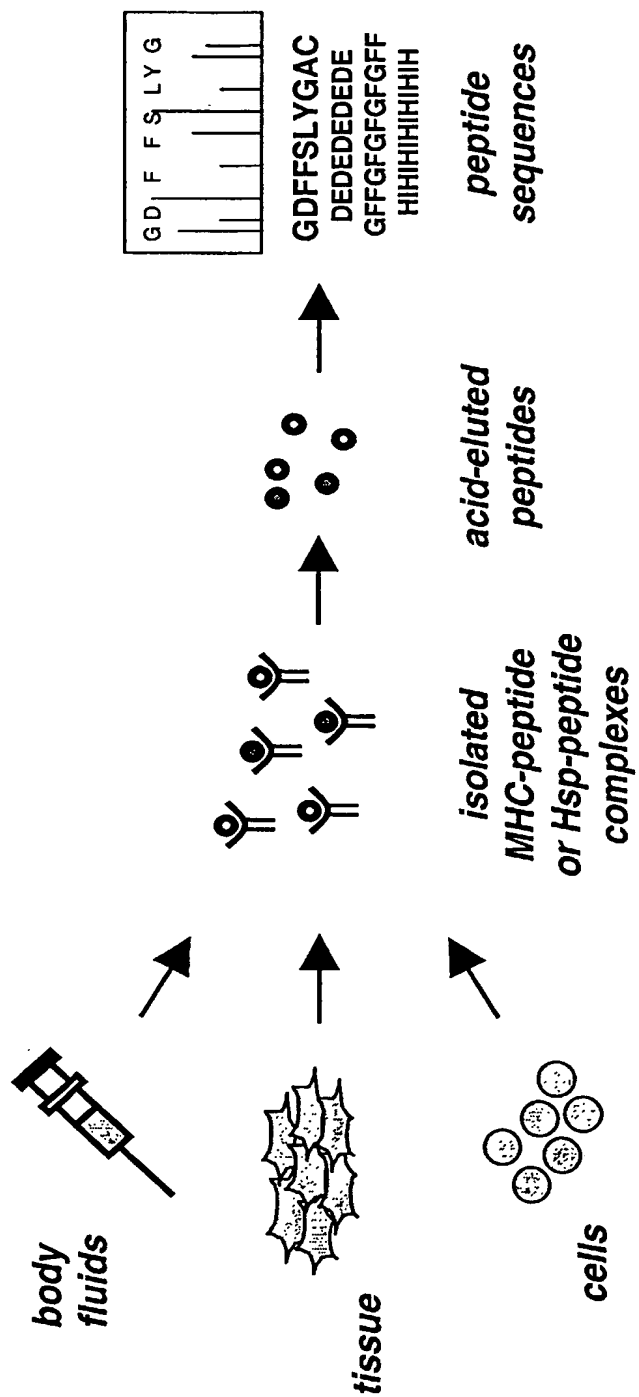
body
fluids

tissue

cells

isolated
MHC-peptide
or Hsp-peptide
complexes

acid-eluted
peptides

GDFFSLYGAC
DEDEDEDE
GFFGFGFGFF
HIHIHIHIHIH

peptide
sequences

GD F F S LY G

Fig. 1A

KLKLKLKLKL
MNMMNMNM
PQPQPQPQP

MNMNMNMN
PQPQPQPQP

peptide
sequences

acid-eluted
peptides

isolated
HLA-peptide

Mature
DC

+ TNFα

+ TNFα

antigenic
source

Immature
DC
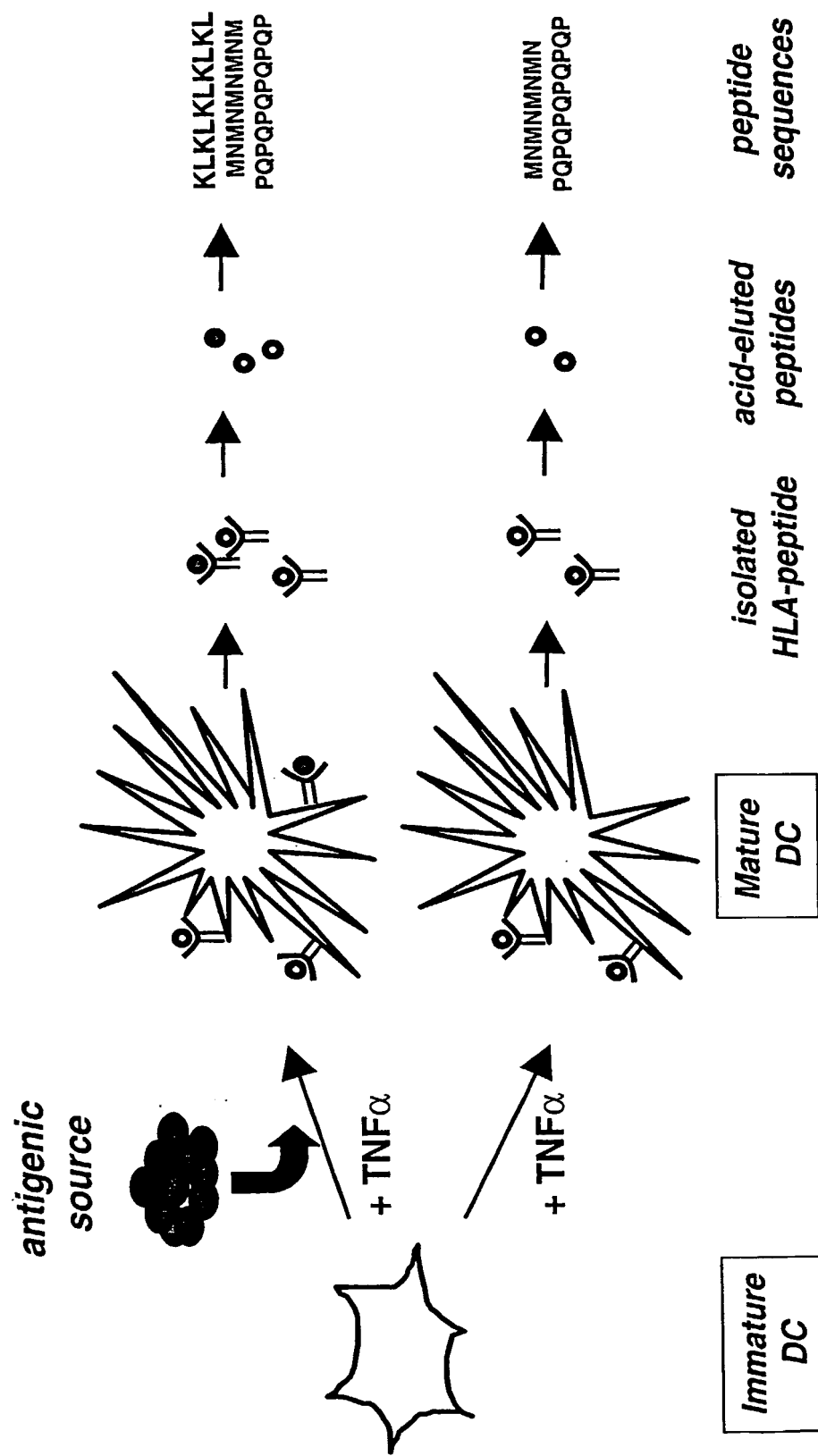
Fig. 1B
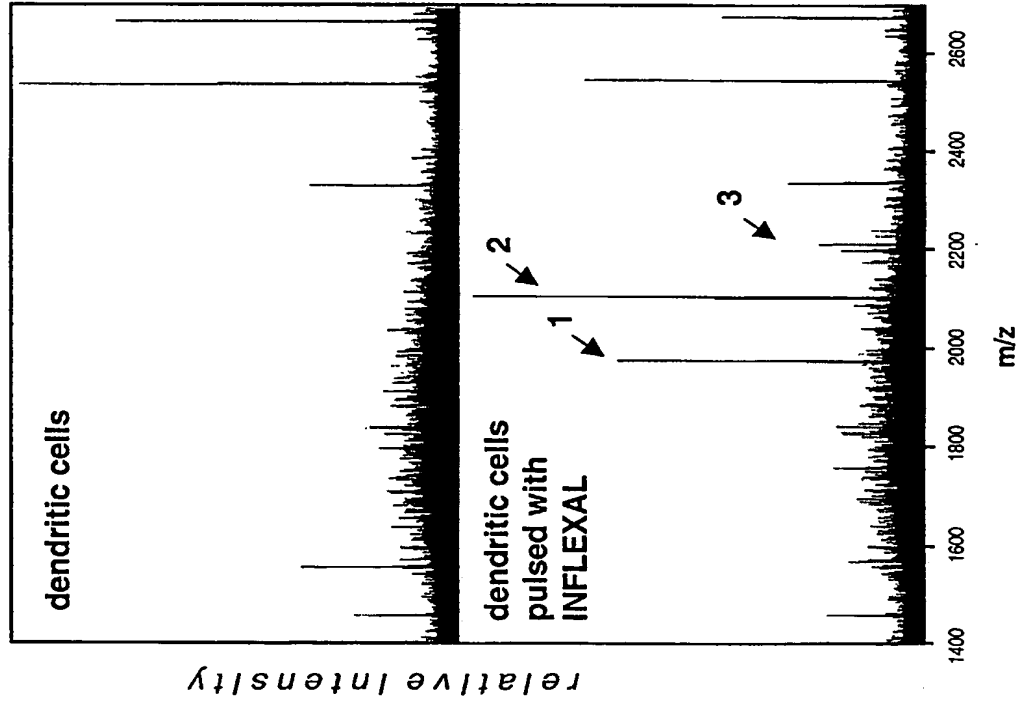
nr	epitope	sequence	mass
1	HA(254-271)	PGKTGTIVYQRGILLPQK	1969.4
1	HA(253-270)	KPGKTGTIVYQRGILLPQ	1969.4
2	HA(253-271)	KPGKTGTIVYQRGILLPQK	2097.6
3	HA(253-271)	KPGKTGTIVYQRGILLPQKV	2196.6

Fig. 2A

Influenza hemagglutinin (HA)
strain B/Yamanashi/166/98

```
         10          20          30          40          50
          |           |           |           |           |
DRICTGITSS NSPHVVKTAT QGEVNVTGVI PLTTTPTKSH FANLKGTKTR

         60          70          80          90         100
          |           |           |           |           |
GKLCPTCLNC TDLDVALGRP MCVGVTPSAK ASILHEVRPV TSGCFPIMHD

        110         120         130         140         150
          |           |           |           |           |
RTKIRQLPNL LRGYEKIRLS TQNVINAEKA PGGPYRLGTS GSCPNATSRS

        160         170         180         190         200
          |           |           |           |           |
GFFATMAWAV PKDNNKTATN PLTVEVPHIC TKEEDQITVW GFHSDNKTQM

        210         220         230         240         250
          |           |           |           |           |
KNLYGDSNPQ KFTSSANGVT THYVSQIGGF PDQTEDGGLP QSGRIVVDYM

        260         270         280         290         300
          |           |           |           |           |
VQKPGKTGTI VYQRGILLPQ KVWCASGRSK VIKGSLPLIG EADCLHEKYG

        310         320         330         340        346
          |           |           |           |           |
GLNKSKPYYT GEHAKAIGNC PIWVKTPLKL ANGTKYRPPA KLLKER
```

Fig. 2B

dendritic cells

dendritic cells
pulsed with
necrotic
melanoma cells

1820.6

relative intensity

m/z

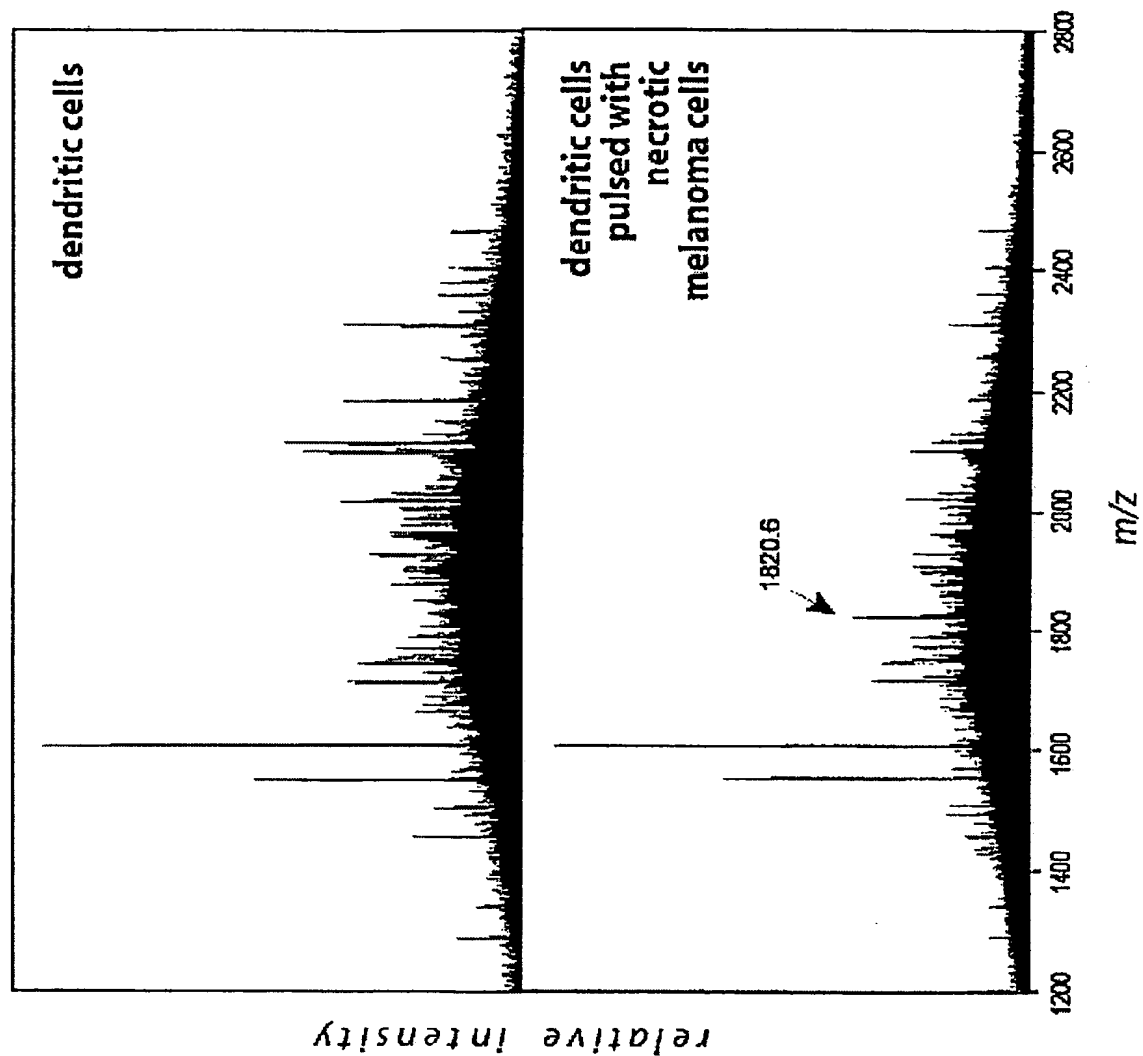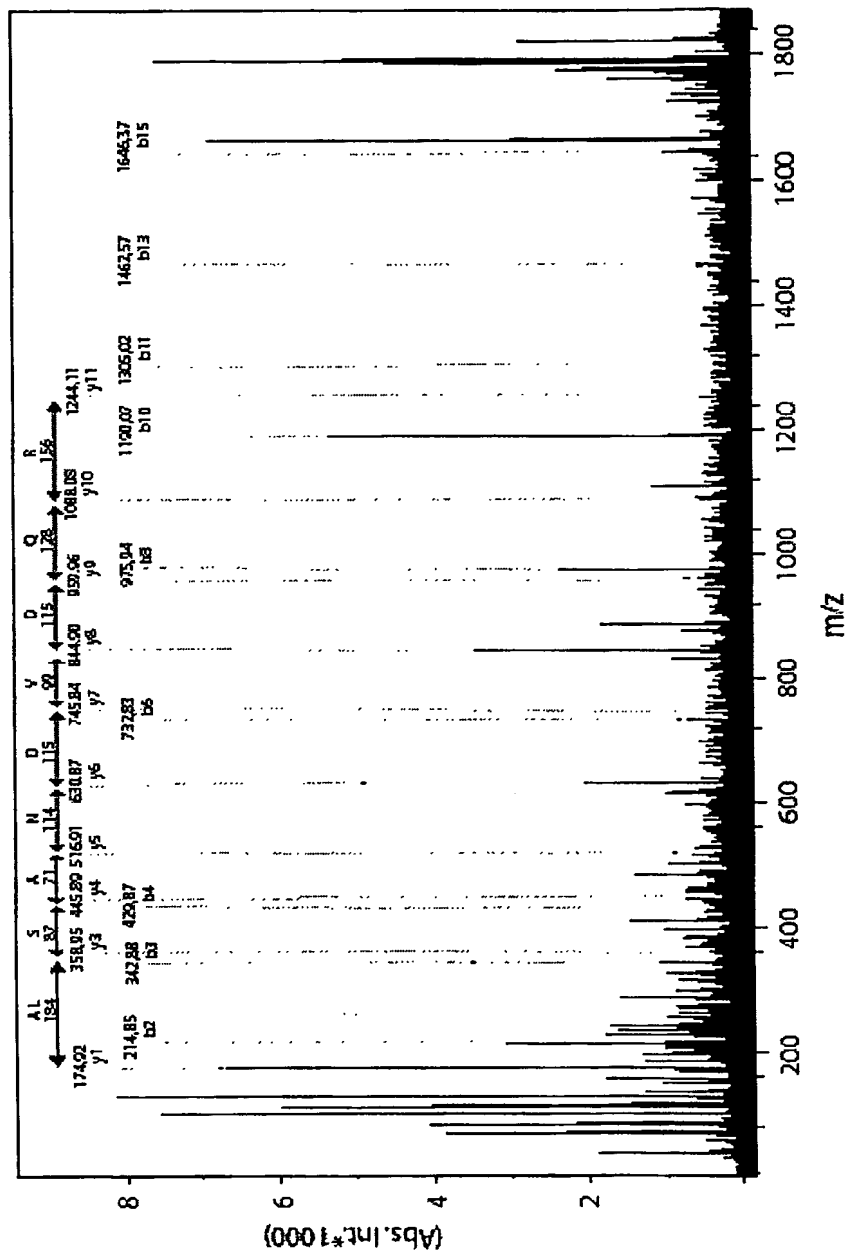1200   1400   1600   1800   2000   2200   2400   2600   2800

Fig. 3A

MALDI-PSD Fragmentation Spectrum

Fig. 3B

ION-TRAP MS-MS Spectrum

Fig. 3C